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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

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Additional inventors are being named on the 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

HUMAN CATHELICIDIN ANTIMICROBIAL PEPTIDES

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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☐ Application Date Sheet. See 37 CFR 1.76

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☑ Applicant claims small entity status. See 37 CFR 1.27.

☑ A check or money order is enclosed to cover the filing fees.

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Respectfully submitted,

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PROVISIONAL APPLICATION COVER SHEET
Additional Pag

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Docket Number 6627-PR4043

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HUMAN CATHELICIDIN ANTIMICROBIAL PEPTIDES

BACKGROUND OF THE INVENTION

Peptides with antimicrobial activity are found throughout nature and are known to be important in the defense of plants insects and animals. Peptides are present predominantly on skin and in the digestive tract as a preliminary form of defense. Many antimicrobial peptides display broad spectrum antimicrobial activity against both Gram-negative and Gram-positive bacteria, fungi and viruses. A number of families of antimicrobial peptides are listed in the attached figures and manuscript. Humans with inability to increase cathelicidins and defensins, two human antimicrobial peptides, in response to inflammatory stimuli develop atopic dermatitis. Genetic disorders such as Kostmann syndrome suffer from frequent infections and neutrophil dysfunction due to a deficiency in the production and processing of cathelicidins.

Most antimicrobial peptides are synthesized as inactive peptides that are activated by proteases to release active peptides. An inability to process the peptides results in disease. A lack of processing of alpha defensins from Panath cells of Matrilysin deficient mice leads to impaired clearance of infection in the gut. Inhibition of the proteases that are involved in processing of the inactive peptides impairs bacterial clearance in wounds. Cleavage results in the generation of other active peptides that function to stimulate other processes such as chemotaxis and angiogenesis.

SUMMARY OF THE INVENTION

The invention is the discovery of a series of active cathelicidin peptide fragments isolated from human sweat. The peptides are demonstrated to be fragments of cathelicidin by the use of antibodies and by amino acid sequencing of a portion of the peptides. The peptides have been demonstrated to have antimicrobial activity by antimicrobial assay

and low toxicity by a hemolysis assay. As the peptides are naturally occurring, the chance of adverse reaction to the peptides is minimal. The peptides have been shown to have substantially reduced pro-inflammatory activity known to be present in full length cathelicidin antimicrobial peptides. Moreover, the absence of resistant strains to these natural defenses of the body strongly suggests that mutations to create resistance to the antimicrobial peptides is detrimental to the viability of microbial pathogens.

The invention is the use of the antimicrobial peptides or fragments thereof isolated from sweat as antimicrobial agents for the prevention and/or treatment of infection in an organism including a human, other animal or plant. Preferably, the peptides are applied topically. In a human or other animal, the peptides can also be administered by other routes such as oral and parenteral. Additionally, the peptides can be used for decontamination of surfaces. The peptides may be used alone or in combination with each other. The peptides may be synthesized individually and combined. Alternatively, larger portions of the cathelicidins can be synthesized, treated with an appropriate protease to generate a mixture of antimicrobial peptides and administered to an organism or applied to a surface.

The invention is a method of activation of the antimicrobial peptides of the instant invention comprising incubation of the antimicrobial peptides in a bicarbonate buffer containing between about 15 mM to about 500 mM bicarbonate, preferably about 50 mM, preferably sodium bicarbonate. The bicarbonate buffer can also be used to modulate the solubility properties of the peptides for ease of application or administration.

The invention is further related to the compositions for use in the instant invention. The compositions include the peptides themselves as well as the peptides in the carbonate buffer of the invention.

The invention is a method for the identification of novel

antibacterial peptides comprising incubation of antimicrobial peptides with sweat for sufficient time to allow for proteolysis, isolation of peptide fragments and assaying for antimicrobial activity.

The invention is related to small molecule analogs or stabilized peptides based on the peptides of the invention or peptides identified by methods of the invention. Peptides can be modified for any of a number of reasons such as to improve phamacokinetic and pharmacodynamic properties.

BRIEF DESCRIPTION OF THE DRAWINGS

TABLE 1. Antimicrobial and hemolytic activity of human sweat peptides.

TABLE 2. Cathelicidin peptide antimicrobial function screen.

FIG. 1. Soluble antimicrobial activity on human skin.

FIG. 2. Serine protease in sweat enhances cathelicidin antimicrobial activity.

FIG. 3. Purification of novel cathelicidin peptides generated from LL-37.

FIG. 4. Identification of novel cathelicidin peptides generated from LL-37.

FIG. 5. Processing of LL-37 enhances antimicrobial activity.

FIG. 6. Processing of LL-37 decreases ability to stimulate keratinocyte IL-8

FIG. 7. Percent inhibition of Staph Aureus by LL-37 in 100 mM NaCl with or without bicarbonate buffer, 10% FCS- 2 hours to overnight.

FIG. 8. Growth of Staph Aureus in 100 mM NaCl, 10% FCS media containing various $NaHCO_3$ concentrations.

FIG. 9. Carbonate containing compounds increase the antimicrobial activity of LL-37.

FIG. 10. Bicarbonate itself, not pH, is responsible for antimicrobial enhancement.

FIG. 11. Bicarbonate enhances the activity of cathelicidin antimicrobial peptides.

FIG. 12. 25mM $NaHCO_3$ enhances LL-37 activity without effecting bacterial growth.

FIG 13. $NaHCO_3$ enhances antimicrobial activity against gram negative and gram positive bacteria.

FIG. 14. $NaHCO_3$ enables direct membrane disruption of Staph aureus.

FIG. 15. $NaHCO_3$ enhances the activity of various antimicrobial peptides.

FIG. 16. Electron microscopy reveals bacterial cell wall lysis by LL-37 and bicarbonate.

FIG. 17. Antimicrobial peptides and innate defense of the skin (40 pages)

FIG. 18. Antimicrobial peptides and innate defense of the skin (40 pages)

FIG. 19. Antimicrobial peptides and innate defense of the skin (37 pages)

The present invention will be better understood from the following detailed description of an exemplary embodiment of the invention, taken in conjunction with the accompanying drawings.

DETAILED DESCRIPTION OF THE DRAWINGS

FIG. 1. Soluble antimicrobial activity on human skin. Human sweat was concentrated 50X and separated by HPLC on C18. a) Absorbance profile at 214 nm for eluted material from 35% to 60% acetonitrile, inset: complete absorbance profile of eluted materials. b) Ability of material eluted in FIG. 1 a to inhibit growth of *S. aureus mprF* is shown as diameter of zone of *S. aureus mprF* inhibition. Several antimicrobial fractions were detected. Mass spectrometry identified 3 previously described antimicrobials; fractions

labeled (1) and (3) are dermcidin and DCD-1L respectively (MW 4701, MW 4818, respectively) confirmed by N-terminal sequencing ((1): SSLLEKGLDGA, (3): SSLLE,). (2): LL-37 identified by mass spectrometry (MW 4493) and immunoblot. Data representative of single experiment repeated 5 times with separate sweat preparations.

FIG. 2. Serine protease in sweat enhances cathelicidin antimicrobial activity. Antibacterial activity evaluated by radial diffusion assay against *S. aureus mprF* after incubation of LL-3 7 (32μM) in sweat. a) Increase in the inhibition zone is seen when incubated in sweat but not 10 mM PBS or distilled water (DW). b) Diameters of inhibition zone after 6hr incubation at 37°C in (SWT buffer; sweat buffer salts alone), (PBS), (D.W.; distilled water), (SWT; sterile filtered human sweat at 37°C), (SWT at 4°C), (SWT with P1, with protease inhibitor cocktail). c) Action of specific protease inhibitors on gain of antimicrobial activity. Data show increase in inhibition zone at 37°C after 6hr compared to 0 hr. Serine protease inhibitors AEBSF and Aprotinin were most effective. N.E.I. = neutrophil elastase inhibitor, L.E.I.= leukocyte elastase inhibitor. Data are triplicate determinations ±SEM from single experiment representative of 3.

FIG. 3. Purification of novel cathelicidin peptides generated from LL-37 a) Human sweat was separated by HPLC on C18. Absorbance profile at 214 nm is shown for eluted material from 30% to 60% acetonitrile. Crude is sweat prior to addition of LL-37, overlay plots show separate runs of sweat following addition of 32nmoles of LL-37 and incubation for 0 to 24 hr at 37°C. b) Antibacterial activity with radial diffusion assay against *S. aureus mprF* of fractions eluted from 24hr sample in a. Peaks with antimicrobial activity are labeled 1 and 2 and 3.

FIG. 4. Identification of novel cathelicidin peptides generated from LL-37. Following purification shown in Figure 3, major bioactive fractions were identified. a) peak (1) in Fig3a identified as KR-20 by mass spec. (MW 2468), and N-terminal sequence KRIVQRIKDVF, b) peak (2) detected 2 peptides; RK-3 1 and KS-30 (MW 3647, 3803, and RKSKEKTG, KSKEKTGK, respectively), c) Western blot analysis with anti-LL-37 was done on all

fractions from Fig 3, shown are fractions eluting at acetonitrile concentrations 46-55. Peaks labeled (2) and (3) in Figure 3 were immunoreactive. d) peak (3) identified as LL-37 (MW 4493). Data from single experiment representative of 3.

FIG. 5. Processing of LL-37 enhances antimicrobial activity. The antimicrobial activity of cathelicidin peptides was evaluated by radial diffusion assay against (a) *S. aureus*, (b) *E. coil,* (c) solution assay against *C. aibicans* and (d) Group A Streptococci. The antimicrobial activity of each peptide was evaluated in 10%TSB/ 10mM phosphate buffer with several NaCl concentraions against *E.coii* (e) and *S.aureus* (f). Data shown are representative of triplicate determinations.

FIG. 6. Processing of LL-37 decreases ability to stimulate keratinocyte IL-8. Cathelicidin peptides were added to culture of normal human keratinocytes at a final concentration of 3 or 10 μM then IL-8 release determined after 8 hr. All samples were endotoxin free by limulus assay. Data are mean ±SEM of triplicate determinations.

FIG. 7. Percent inhibition of Staph Aureus by LL-37 in 100 mM NaCl with or without bicarbonate buffer, 10% FCS- 2 hours to overnight. Inhibition of growth of *S. aureus* by LL-37 with or without activation with carbonate anion was tested. As can be seen, the presence of 50mM $NaHCO_2$ in the buffer substantially inhibited the growth of *S. aureus*.

FIG. 8. Growth of Staph Aureus in 100 mM NaCl, 10% FCS media containing various $NaHCO_3$ concentrations. A number of concentrations of $NaHCO_3$ were tested to determine the concentration required to activate the LL-37 without being toxic to the bacteria. As can be seen, killing of bacteria by ll-37 is greatly enhanced in the presence of 25 mM $NaHCO_3$. At higher concentrations (e.g., 100 mM $NaHCO_3$) the effects of the bicarbonate on the activation of LL-37 are difficult to determine due to the apparent toxicity of the bicarbonate to the cells. Optimization of the concentration of bicarbonate to activate antimicrobial peptides is well within the ability of those skilled in the art.

FIG. 9. Carbonate containing compounds increase the antimicrobial

activity of LL-37. Carbonate was identified as the antimicrobial enhancing factor in mammalian tissue culture media via serial deletion of Minimal Essential Media (MEM) components. Staph aureus was cultured in 20% Tryptic Soy Broth (TSB), 10% Fetal Bovine Serum (FBS) and 70% of various media or media components with, or without, 32μM LL-37 to assess which components could increase antimicrobial activity. MEM buffers are 2200 mg/L $NaHCO_3$, 140 mg/L NaH_2PO_4 and 6800 mg/L NaCl. MEM salts are 200mg/L $CaCl_2$, 400 mg/L KCl, 98 mg/L $MgSO_4$. All solutions were adjusted to pH 7.4. Bacterial growth was measured as the change in turbidity at OD600 and % inhibition was determined by comparing bacterial growth in the presence of peptide to that in the absence of peptide. 32μM LL-37 inhibited Staph aureus growth poorly in TSB media alone, or with NaCl and FBS. In the presence of MEM buffers, inhibition was greatly increase and $NaHCO_3$, was determined to be the buffering component responsible for this increase. This was confirmed with other carbonate containing compounds, 50mM $KHCO_3$ and Na_2CO_3, which also increased antimicrobial activity.

FIG. 10. Bicarbonate itself, not pH, is responsible for antimicrobial enhancement. Inhibition of Staph aureus was measured in 20% TSB, 10% FBS 1mM NaH_2PO_4, 150mM NaCl, with either 50mM $NaHCO_3$ or 50mM Glycine buffer at various pH. Only bicarbonate containing media was able to confer increased antimicrobial activity to LL-37.

FIG. 11. Bicarbonate enhances activity of cathelicidin antimicrobial peptides. Inhibition of Staph aureus growth in the presence of various concentrations of human (LL-37), murine (CRAMP) and porcine (PR-39) Cathelicidin antimicrobial peptides was measured in 20% TSB, 1mM NaH_2PO_4, with, or without, 50mM $NaHCO_3$ at pH 7.4. The presence of $NaHCO_3$ greatly reduced the minimal inhibitory concentration (MIC) of all three peptides.

FIG. 12. 25mM $NaHCO_3$enhances LL-37 activity without effecting bacterial growth. The growth of Staph aureus in the presence, or absence, of 32 μM LL-37 was measured in 20% TSB, 10% FBS, 1mM NaH_2PO_4, 150mM NaCl, and various concentrations of $NaHCO_3$, all at pH 7.4. 25mM $NaHCO_3$

yielded complete inhibition of bacterial growth in the presence of LL-37, but not without peptide. High concentrations of $NaHCO_3$ retarded the growth of Staph aureus.

FIG. 13. $NaHCO_3$enhances antimicrobial activity against gram negative and positive bacteria. Staph aureus and E. coli were resistant to 16μM CRAMP without 50mM $NaHCO_3$, but became susceptible in its presence in 20% TSB, 1mM NaH_2PO_4 at pH 7.4. Group B Strep (GBS) and Salmonella were susceptible even without $NaHCO_3$.

FIG. 14. $NaHCO_3$ enables direct membrane disruption of Staph aureus. Release of cytoplasmic expressed beta-galactosidase was measure from bacteria with and without the addition of $NaHCO_3$. A) polymyxin B induces greater leakage of cytoplasmic protein with carbonate. B) LL-37 induces greater leakage of cytoplasmic protein with carbonate

FIG. 15. $NaHCO_3$ enhances the activity of various antimicrobial peptides. The ability of 50mM $NaHCO_3$ (EF) to enhance the activity of diverse antimicrobial peptides against Staph aureus was tested in 20% TSB, 10% FBS, 1mM NaH_2PO_4, 150mM NaCl, pH 7.4. The activity of all 3 Cathelicidins, Cryptdin-4 and Human Beta Defensin-2 (HBD-2) were enhanced by the addition of bicarbonate. Several other antimicrobial peptides were not enhanced.

FIG. 16. Electron microscopy reveals bacterial cell wall lysis by LL-37 and bicarbonate. Staph aureus was grown for four hours in 20% TSB, 1mM NaH_2PO_4 with or without 25mM $NaHCO_3$or 32μM LL-37. Bacteria were fixed in Karnovsky's Solution 24 hours and processed for EM on a Zeiss EM 10B Transmission Electron Microscope by the VA Microscopy Core Facility. The presence of 25mm $NaHCO_3$ alone had no visible effect on the bacterial cell wall. 32 uM LL-37 caused a small amount of damage to bacterial cell walls in the absence of $NaHCO_3$, but totally ablated them in its presence, causing cell lysis. Mag 42,000X, insets are 110,000X.

Although an exemplary embodiment of the invention has been described above by way of example only, it will be understood by those

skilled in the field that modifications may be made to the disclosed embodiment without departing from the scope of the invention, which is defined by the appended claims.

WE CLAIM:

CLAIMS

1. A method of prevention or treatment microbial infection in an organism comprising administration of an antimicrobial peptide or fragment thereof to the organism.

2. The method of claim 1, wherein the microbial infection is caused by an organism selected from the group consisting of gram positive bacteria, gram negative bacteria, fungus and virus.

3. The method of claim 1, wherein the antimicrobial peptide or fragment thereof is selected from the group consisting of bombinins, cecropins, dermaseptins magainins, defensins, protegrins and cathelicidins.

4. The method of claim 1, wherein the antimicrobial peptide or fragment thereof is a cathelicidin.

5. The method of claim 4, wherein the fragment of cathelicidin comprises a peptide of at least 4 amino acids in length.

6. The method of claim 4 wherein the fragment of cathelicidin comprises a peptide of at least 4 amino acids in length from a mature amp domain of the cathelicidin.

7. The method of claim 4, wherein the fragment of cathelicidin is selected from the group consisting of LL-37, LL-25, LL-20, LL-15, LL-12, GD-23, RK-19, EK-20, VQ-17, EK-15, GK-17, EF-15, D-EK-20, RRR-20, PR-39, KR-20, RK-30, RK-31, KS-30, DCD-1L, and DCD.

8. The method of claim 4, wherein the fragment of cathelicidin is generated by incubation of substantially a full length cathelicidin with

protease.

9. The method of claim 4, wherein the fragment of cathelicidin is generated by incubation of substantially a cathelicidin amp fragment with protease.

10. The method of claim 1, wherein the antimicrobial peptide or fragment thereof is administered by topical application, oral or parenteral routes of administration.

11. The method of claim 1, wherein the antimicrobial peptide of fragment thereof is combined with a bicarbonate buffer comprising about 15 mM to 500 mM bicarbonate before administration.

12. A method of enhancing activity of an antimicrobial peptide or fragments thereof comprising incubation of the antimicrobial peptides in a bicarbonate buffer system wherein the buffer system comprises a bicarbonate concentration between about 15 mM and 500 mM bicarbonate.

13. The method of claim 12, wherein the antimicrobial peptide or fragment thereof is selected from the group consisting of bombinins, cecropins, dermaseptins magainins, defensins, protegrins and cathelicidins.

14. A composition of matter comprising an antimicrobial peptide or fragment thereof in a bicarbonate buffer system wherein the buffer system comprises a bicarbonate concentration between about 15 mM and about 500 mM bicarbonate.

15. The composition of claim 14, wherein the antimicrobial peptide

or fragment thereof is selected from the group consisting of bombinins, cecropins, dermaseptins magainins, defensins, protegrins and cathelicidins.

16. A method of decontamination of a surface comprising application of an antimicrobial peptide or fragment thereof to the surface.

17. A method for the identification of novel antimicrobial peptides comprising, incubation of antimicrobial peptides with sweat for sufficient time to allow for proteolysis, isolation of peptide fragments and assaying for antimicrobial activity.

ABSTRACT

The production of antimicrobial peptides is essential for the defense against infection. The invention is the discovery of a number of antimicrobial peptides in human sweat. The invention is a method of prevention and treatment of microbial infections comprising the use of the peptides. The invention is a method of activation of microbial peptides comprising incubation with bicarbonate buffer. The invention is a method for the discovery of new antimicrobial peptides comprising incubation of antimicrobial peptides with sweat, isolation of antimicrobial peptide fragments and testing them for the presence of antimicrobial activity. The invention is the development and use of peptide analogs of the antimicrobial peptides disclosed in the application or identified using the method of the invention.

Post-secretory processing generates multiple cathelicidins for enhanced topical antimicrobial defense[1]

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Abstract:

The production of antimicrobial peptides is essential for defense against infection. Many of the known human antimicrobial peptides are multifunctional, with stimulatory activities such as chemotaxis while simultaneously acting as natural antibiotics. In humans, eccrine appendages express DCD and CAMP, genes encoding proteins processed into the antimicrobial peptides Dermcidin and LL-37. Here we show that after secretion onto the skin surface, the CAMP gene product is processed by a serine protease-dependent mechanism into multiple antimicrobial peptides previously unknown and distinct from the cathelicidin LL-37. These peptides show enhanced antimicrobial action, acquiring the ability to kill skin pathogens such as *Staphylococcus aureus* and *Candida albicans*. Furthermore, although LL-37 may influence the host inflammatory response by stimulating IL-8 release from keratinocytes, this activity is lost in subsequently processed peptides. Thus, a single gene product encoding an important defense molecule alters structure and function in the topical environment to shift the balance of activity towards direct inhibition of microbial colonization.

Introduction:

Peptides with antimicrobial activity are found throughout nature and are known to be important for the immune defense of plants, insects and animals(1). Many of these peptides exhibit a broad spectrum of antimicrobial activity, inhibiting or killing Gram-positive and Gram-negative bacteria, fungi and viruses. In mammals, antimicrobial peptides belonging to the cathelicidin family have been shown to be important for the antimicrobial efficacy of neutrophils, macrophages, and mast cells (2-6). In addition, epithelia of lung, gut, urinary bladder, oral mucosa and skin produce antimicrobial peptides of the defensin and cathelicidin family constitutively or in response to injury (7-9). At the epithelial interface with the external environment, these molecules are thought to serve as a rapid first line defense for inhibition of microbial proliferation and invasion.

In mammalian skin, cathelicidins have been directly shown to be essential for defense against invasive bacterial infection by Group A Streptococcus(10). Found in abundance in neutrophils and mast cells, the cathelicidins are expressed at relatively low levels in normal keratinocytes but are rapidly induced during inflammation(11). The recruitment of cathelicidin-rich cells, and increase in expression by the epithelial keratinocytes, leads to accumulation of cathelicidins in wound fluid and in the overlying crust(12). Recently, cathelicidins and dermcidin have also been found constitutively produced by the eccrine apparatus and secreted constitutively into human sweat (13, 14). In this scenario, antimicrobial activity becomes available at the most external interface, providing an inhibitory barrier to infection.

The clinical consequences of antimicrobial peptide expression are seen by observations that patients with atopic dermatitis lack the ability to increase cathelicidins and defensins in response to inflammatory stimuli(15). Since patients with atopic dermatitis are uniquely susceptible to infection when compared to normal individuals or those with elevated expression as seen in psoriasis, this first line immune defense mechanism appears to have an essential function for resistance against skin infections. Similarly, patients with Kostmann syndrome, a rare inherited disorder characterized by frequent infections and neutrophil dysfunction, have a deficiency in production and processing of

cathelicidin (16). Such emerging clinical associations support the need to further explore the function and regulation of this evolutionarily ancient aspect of the human immune system.

Most antimicrobial peptides are synthesized as inactive preproproteins that require enzymatic processing for release of active peptides. Lack of processing, such as seen in alpha defensins from Panath cells of Matrilysin deficient mice(17), leads to impaired clearance of infection in the gut. Similarly, inhibition of processing of porcine cathelicidins by elastase impairs bacterial clearance in wounds(18). The enzymatic processing of cathelicidins from a pro-protein to a two-component solution consisting of the cathelin prodomain and the C-terminal cationic antimicrobial peptide, LL-37, can have multiple consequences for immune defense. The cathelin domain is itself antimicrobial and functions to inhibit cysteine proteases such as Cathepsin L (19). LL-37, while functional as a broad spectrum antimicrobial, can also stimulate chemotaxis and angiogenesis by binding formyl peptide receptor-like-1 (FPRL-1) (20, 21) and improve reepithelialization to enhance wound repair(22).

In this study we sought to determine if additional antimicrobial peptides are present in the most superficial barrier topically provided by human sweat. Our findings suggest that human cathelicidin is further processed and enables a shift in biological activity towards antimicrobial function and away from the ability to stimulate host response.

Materials and Meth ds

Sweat collection and processing

Sweat was collected on paper tissues (Kimwipes, Kimberly-Clark, GA) from healthy volunteers after exercise as previously described(13). After collection, 20 ml of sweat was centrifuged at 2000g for 15 min. at 4°C, filtered through a 0.20 µl filter (Acrodisc syringe filter, 0.2 µm, low protein binding, Fisher scientific, Tustin, CA), and frozen at –80°C. For some experiments sweat was lyophilized to dryness, then suspended in 400 µl of distilled water (DW: cell culture grade, endotoxin free, GibcoBRL, Grand island, NY). For analysis of LL37 processing by sweat, 1.6 nmol of LL-37 synthetic peptide was incubated in 50 µl of sweat for 0, 1, 6, 24 hrs at 37 °C or 4°C. In some experiments proteinase inhibitors including; mixed protease inhibitor cocktail (1tab/10ml, Roche, Indianapolis, IN), 100 µg/ml Bestatin, 10 µg/ml E-64, 10 µg/ml Aprotinin, (Sigma, St Louis, MO); 100 µM AEBSF, 100 µM Neutrophil elastase inhibitor (NEI) or 100 µM leukocyte elastase inhibitor (LEI) (Calbiochem, San Diego, CA) were added during incubation. After incubation, 2 µl was assayed by radial diffusion assay to determine antibacterial activity. For analysis by HPLC 32 nmoles LL-37 was incubated in 100ul. To control for potential contamination eluted from paper tissues, parallel processing was done on tissues soaked in phosphate-buffered saline (PBS:137 mM NaCl, 2.7 mM KCL, 4.3 mM Na_2HPO_4-H_2O, 1.4 mM KH_2PO_4, pH 7.4). No antimicrobial activity was detectable in these preparations. Protein concentrations were evaluated by BCA assay (protein assay reagent, Pierce, Rockford, IL) or Bradford protein assay (Bio-Rad, Hercules, CA) according to manufacture's instructions. Human tissue and blood collection was approved by the UCSD Human Research Protection Program.

Peptide synthesis

Dermcidin, LL-37, RK-31, KS-30, and KR-20 peptides were commercially prepared by Synpep Corporation, Dublin, OR. Peptide amino acid sequences were LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES (LL-37), RKSKEKIGKEFKRIVQRIKDFLRNLVPRTES (RK-31), KSKEKIGKEFKRIVQRIKDFLRNLVPRTES (KS-30), KRIVQRIKDFLRNLVPRTES (KR-20), SSLLEKGLDGAKKAVGGLGKLGKDAVEDLESVGKGAVHDVKDVLDSV (dermcidin). All

synthetic peptides were purified by HPLC and identity confirmed by mass spectrometry.

HPLC chromatography

Peptide separation was performed using an AKTA purification system (Amersham Pharmacia Biotech, Piscataway, NJ) on a Sephasil peptide C18 column (12 μm, ST 4.6/250, Amersham Pharmacia Biotech, Piscataway, NJ). Concentrated human sweat, or LL-37 incubated in sweat, was separated by reversed phase (RP-HPLC) following column equilibration in 0.1% TFA at a flow rate of 0.5 ml/ml and eluted using gradients of 0-35% and 35-60% acetonitrile for 16 min, 67 min, or 0-60% acetonitrile for 120 min. Column effluent was monitored at 214, 230, and 280nm. All collected fractions (1 ml) were lyophilized and suspended in 10 μl of DW for antimicrobial radial diffusion assay.

Western and Immunoblot analysis

Fractions purified by HPLC as described above were evaluated by quantitative dot-blot and Western Blot. 2 μl of each fraction was compared to a standard curve of synthetic LL37 peptide applied onto PVDF membrane (Immobilon-P, Millipore, MA). Antibody used was rabbit anti-LL-37 polyclonal antibody derived and affinity purified against the entire LL-37 peptide. For immunoblot, membranes were blocked (0.1% TTBS: 5% nonfat milk in 0.1% Tween 20 / tris buffered saline (TBS: 150 mM NaCl, 10 mM Tris Base, pH 7.4)) for 60 min at R.T., and then rabbit anti LL37 polyclonal antibody (1:5000 in blocking solution) was incubated with the membrane overnight at 4°C. After washing 3x with 0.1% TTBS, horseradish peroxidase labeled goat anti rabbit antibody (1:5000 in the blocking solution, (DAKO, CA) was incubated with the membrane for 60 min, R.T. After washing the membrane again with 0.1% TTBS, the membrane was immersed in ECL solution (Western Lightning Chemiluminescence Reagents Plus, New Lifescience Products, Boston, MA) for 60 sec then exposed to X-ray film (Kodak). For, Western-blot analysis, sweat samples (10 μl) were separated by 16.5% tris-tricine / peptide gel (BIO-RAD, Hercules, CA), and then transferred on to a PVDF membrane (Immobilon-P, Millipore, MA). For positive control, 5 pmol LL-37 synthetic peptide was applied.

Mass spectrometry and Protein sequence analysis

Mass spectrometry was performed by Center for Mass Spectrometry, The Scripps Research Institute

(La Jolla, CA). MALDI-MS spectra were obtained with a Voyager DE-RP MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a nitrogen laser (337 nm, 3-ns pulse). Spectra were collected in reflector mode. The accelerating voltage in the ion source was 20 kV. Data were acquired with a transient recorder with 2-ns resolution. The matrix used in this work was -cyano-4-hydroxycinnamic acid dissolved in water/acetonitrile (1:1, v/v) to give a saturated solution at room temperature. To prepare the sample for analysis, 1 µl of the peptide solution (containing 1-10 pmol of protein in 0.1% trifluoroacetic acid) was added to 1 µl of the matrix solution and applied to a stainless steel sample plate. The mixture was then allowed to air dry on the sample plate before being introduced into the mass spectrometer. Each spectrum was produced by accumulating data using 128 laser pulses. Mass assignments were assigned with an accuracy of approximately ± 0.1% (± 1 Da/1000 Da). Protein sequence analysis for target HPLC fractions was performed by Division of Biology Protein Sequencer Facility, University of California, San Diego. The amino acid sequencing was performed on an Applied Biosystems Procise Model 494 sequencer using the "pulsed-liquid" program supplied by the manufacture.

Antimicrobial assays

For screening of antimicrobial activity of HPLC fractions, radial diffusion assay was used as previously described(13). Lyophilized HPLC fractions were dissolved in DW (GibcoBRL, Grand Island, NY), and tested against *Staphylococcus aureus mprF*. Thin plates (1 mm) of 1% agarose in 0.5% tryptone containing approximately $1x10^6$ cells/ml of *S. aureus mprF* were used. 1 mm wells were punched in the plates and 2µl of samples dissolved in tissue culture grade sterile water were loaded in each well. As a positive control, synthetic LL-37 was applied to separate wells. After incubation at 37 °C overnight, the inhibition zones were recorded by CCD camera and diameters measured.

To evaluate antimicrobial activity against wild-type *S. aureus* (clinical isolate), and enteroinvasive *E.coli* O29, both radial diffusion and solution killing assays were done. Radial diffusion assays were done as described for *S.aureus mprF*. Solution killing was done in 10%TSB in 10mM PB (TSB= 30gm/L Tryptic Soy Broth, Sigma, St Louis MO; 20X PB= 27.6 g/L NaH_2PO_4-H_2O, 53.65 g/L Na_2HPO_4-$7H_2O$, pH 7.4). Bacteria in log-phase growth were suspended to $1X10^6$ cells/ml, peptide

added and incubated at 37°C for 2 hr. Bacteria were then plated on TSB agar (TSB, Bactoagar 13g/L, Becton Dickinson, Sparks MD) for direct colony count and determination of CFU. Activity against Group A Streptococcus (NZ131) was done only in solution assay as described. To evaluate antimicrobial activity in high salt conditions, solution assay was done in 10%TSB/10mM PBS with several salt concentrations (concentration of NaCl; 10, 50, 100, 150, 300, 500 mM). Action against the *C. albicans* was determined in Dixon medium (0.6% Peptone, 4% Malt Extract, 1% Glucose, 0.1% Ox Bile, 1% Tween-80) in sterile 96-well microtiter plates (Corning Inc., Corning, NY) at a final volume of 50 µl. The assay mixtures contained 1-2.5 x 10^4 CFU/ml freshly grown Candida, 20% Dixon medium, 0.6mM phosphate buffer pH 7, and 16 µg/ml chloramphenicol. Microtiter plates were incubated at 37°C for 24h with peptides, then plated on Dixon agar to determine the MFC (minimum fungicide concentration).

Hemolysis Assay

Hemolytic activity was determined on human whole blood. Freshly obtained whole blood was washed 3X in PBS and resuspended in PBS at its original volume containing peptides at indicated concentrations. Samples were incubated at 37 °C for 1.5 to 3 h, and hemolysis determined by centrifuging at 300g and measurement of the absorbance of the supernatant at 578 nm. Hemolytic activity of each peptide was expressed as the percentage of total hemoglobin released compared to that released by incubating with 0.1% Triton X-100.

Measurement of IL-8 release from keratinocytes

Normal human keratinocytes (NHK) were cultured in EpiLife cell medium (Cascade Biologics, Portland, OR) containing 0.06 mM Ca^{2+}, 1x EpiLife-defined growth supplement (EDGS), 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen, Carlsbad, CA). Keratinocytes were seeded in a 96-well plate and grown to confluence under standard tissue culture conditions. Cells were incubated with 3µM or 10µM LL-37 or LL37-derived peptides for 8 hours at 37°C. Supernatants were collected and stored at –20°C overnight. IL-8 ELISA Assay was performed according to the manufacturer's instructions (BD OptEIA, Pharmingen, San Diego, CA). Supernatants were diluted 1:10 for assay.

Simultaneously, LDH assays (Roche, Indianapolis, IN) were used to assess the cytotoxicity of peptides to keratinocytes. LDH release following peptide exposure was compared to release induced by 1% Triton X100.

Results

To identify antimicrobial activity present at the skin surface, human sweat was collected from normal volunteers, concentrated, and separated by high performance liquid chromatography (HPLC). Fractions of the material eluted between 35-65% acetronitrile were individually evaluated for the ability to inhibit the growth of *S. aureus mprF* by radial diffusion assay (Figure 1). Multiple distinct fractions were found to be active in this assay. Prior evaluations of human sweat have shown that the antimicrobial peptides LL-37 and dermcidin are produced by the eccrine apparatus and secreted into the topical soluble environment of sweat. As expected, these molecules were detectable in the sweat preparation shown in Figure 1. The presence of LL-37 was confirmed by immunoblot analysis with antibody specific to LL-37 and by MALDI-TOF mass spectrometry. The dermcidin peptides, DCD and DCD-1L, were identified by MALDI-TOF mass spectrometry and N-terminal amino acid sequencing.

Immunoblot analysis with anti-LL-37 antibody of all fractions isolated by HPLC from the human sweat suggested that other molecules related to LL-37 may be present in fractions eluting between 43 and 48% acetonitrile. These fractions were associated with antimicrobial activity but not identifiable by MALDI-TOF mass spectrometry and N-terminal amino acid sequencing from the concentrated sweat preparations. Based on the immunoreactivity and elution profile we hypothesized that these antimicrobial molecules were alternative forms of LL-37 that were further processed in sweat to unique cathelicidin peptides. To test this, human sweat was freshly collected, sterilely filtered, then synthetic LL-37 added to a final concentration of 32 μM. The relative ability of this solution to inhibit the growth of *S. aureus mprF* was then evaluated. Following incubation at 37°C in sweat, LL-37 increased the zone of inhibition and apparent antimicrobial activity (Figure 2a). Incubation of LL-37 under identical conditions in PBS or distilled water did not affect activity. This increase in apparent antimicrobial activity did not occur at 4°C and was inhibited by the addition of protease inhibitor cocktail, thus suggesting the gain in antimicrobial function was a consequence of an enzymatic process (Figure 2b). Addition of specific inhibitors of potential processing enzymes demonstrated that the serine protease inhibitors AEBSF and Aprotinin were most effective in blocking the increase in antimicrobial activity generated by incubation of LL-37 in sweat (Figure 2c).

An increase in the relative ability of LL-37 to inhibit growth of *S. aureus mprF* suggested that the enzymatic processing of this peptide resulted in alternative forms with either increased direct antimicrobial activity or an ability to synergize with the parent peptide. To identify these processed forms of LL-37, synthetic peptide was incubated with the sterile sweat preparation and separated by HPLC after various periods of incubation (Figure 3a). An increase in the relative abundance of several proteins was seen over time. Correlation of this profile with antimicrobial activity showed fractions eluding at 39% and 48% acetonitrile gained antimicrobial activity coincident with and increase in relative abundance of peptides eluting at these positions (Figure 3b). A third major peak of antimicrobial activity seen between 53 and 56% acetonitrile showed a relative decrease in abundance as estimated by absorbance at 214 nm. MALDI-TOF mass spectrometry and N-terminal sequencing of the peptide eluding at 39% acetonitrile identified this as a 20 amino acid cathelicidin derivative, KR-20 (Figure 4a). Similar analysis of peptides eluding at 48% acetonitrile identified two additional cathelicidin peptides RK-31and KS-30 (Figure 4b). Western blot analysis with antibody against LL-37 confirmed antigenic similarity of RK-31 and KS-30 to parent LL-37 (Figure 4c). KR-20 was not detectable with this antibody (data not shown). Material eluting at 55-56% acetonitrile and decreasing in abundance with incubation was identified as LL-37 by both Western blot and Mass spec analysis (Figure 4c & d).

The newly described human cathelicidin peptides KR-20, RK-31 and KS-30 eluded at positions corresponding to unidentified antimicrobial activity seen in crude sweat preparations partially purified in Figure 1. The low relative abundance of these peptides, yet easily detectable antimicrobial activity suggested that these peptides might gain antimicrobial activity with processing when compared to LL-37. To compare the antimicrobial activity of these cathelicidins, purified synthetic peptides corresponding to LL-37, RK-31, KS-30 and KR-20 were assessed by both radial diffusion assay and solution assay against a variety of microbes (Figure 5). RK-31 and KS-30 showed greatly increased action against wild-type *S. aureus* and E. coli. And all three new peptides showed increased fungicidal activity against Candida albicans. Furthermore, these peptides were synergistic, killing bacteria at lower concentrations when present together, (Figure 5d), and maintained activity at increased salt

conditions (Figure 5e,f). Hemolytic activity against human erythrocytes was minimal as seen by assay at a concentration 5 to 10 times greater than that required for antimicrobial activity (Table 1). However, despite gaining antimicrobial activity by processing to shorter forms of the cathelicidin peptide, hemolytic activity of these antimicrobials decreased relative to LL-37.

In addition to the function of antimicrobial peptides as natural antibiotics, many of these molecules have been associated with the ability to stimulate a variety of host responses. To determine if the secretion and processing of cathelicidin peptides at the surface of the skin could also stimulate a host inflammatory response, human keratinocytes were grown in culture and assayed for the release of IL-8 in response to these peptides. All peptide solutions were endotoxin free. LL-37 had a potent ability to stimulate IL-8 release from keratinocytes, but processing to the shorter form of these cathelicidins decreased the ability to stimulate IL-8 (Figure 6). Cellular toxicity of these peptides was simultaneously evaluated by determination of the release of lactate dehydrogenase into keratinocyte culture medium. At a concentration of 3 μM none of the peptides evaluated significantly increased the permeability of keratinocyte membranes (data not shown).

Discussion

Synthesis, processing and release of antimicrobial peptides are essential elements for defense against infection. In epithelia such as skin, oral mucosa, lung and gut, the release of antimicrobial peptides appears to play a particularly important defensive role. However, the expression of antimicrobial peptides by normal skin, an epithelial barrier exposed to constant microbial challenge, is relatively low when compared to mucosal epithelia of other organs. Partial explanation for this apparent inconsistency is found in recent observations that the antimicrobial peptides hCAP 18/LL-37 and dermcidin are produced by eccrine glands and secreted onto the surface of the skin in sweat. This system would provide a mechanism by which a constitutive antimicrobial barrier may form at the skin surface above the permeability barrier of the stratum corneum and in direct contact with the external environment. A problem with this model is that Dermcidin and LL-37 have poor ability to inhibit the growth of important skin pathogens such as *S. aureus* and *Candida albicans*. Because of this limitation, we hypothesized that sweat contains additional antimicrobial peptides with increased activity against relevant skin pathogens. In this study we show that enhanced antimicrobial activity exists in normal human sweat, and is the consequence of processing of LL-37 to previously unknown naturally occurring cathelicidin peptides.

HPLC separation of concentrated sweat samples from different individuals showed remarkable consistency in the elution profile of antimicrobial molecules. Screening assays were done with *S. aureus* mprF, a mutant that lacks the ability to modify anionic membrane lipids with L-lysine(23). This mutation leads to an increase in surface anionic charge and greater binding by cationic host defense molecules. Thus, this strain of *S. aureus* amplifies sensitivity to cationic antimicrobial peptides. Initial purifications confirmed the sensitivity of this approach by detecting peptides already known to occur in human sweat such as LL-37, DCD and it's variant DCD-1L. However, antimicrobial activity associated with less abundant peptides in the crude human sweat preparation was difficult to purify by standard biochemical approaches. Detection of these fractions by antibody against LL-37 suggested that some of these less abundant yet apparently potent antimicrobial molecules were similar to LL-37.

Analysis of antimicrobial activity and identification of new peptides generated from synthetic

LL-37 showed that further processing occurs by a serine protease present in sweat. These previously unknown peptides eluted at positions similar to the antimicrobial activity detected in native sweat, suggesting that RK-31, KS-30 and KR-20 are naturally occurring, but less abundant in sweat immediately after secretion. Unlike the techniques used here to collect sweat for study, under normal conditions sweat remains at the skin surface for further processing and concentration by evaporation. This final enzymatic modification of cathelicidins is thus distinct from that previously studied during neutrophil activation. Bovine and porcine precursor cathelicidins in specific granules are acted upon by elastase in the azurophilic granule to release a single C-terminal antimicrobial peptide(24). In humans, the full-length cathelicidin hCAP-18 can be cleaved to LL-37 by proteinase 3, a protease present in human neutrophils(25). In the vagina at low pH, another serum protease, gastricsin, processes a slightly longer C-terminal peptide (ALL-38) from hCAP-18(26). These systems have not shown further processing of the C-terminal peptide. This may be due to lack of detection of these peptides in prior studies or due to the presence of additional serine proteases at the skin's surface, such as activated tryptases and kallikrein(27). These and/or other serine proteases of host origin, as well as serine proteases potentially released by microflora on the skin, may be responsible for the processing observed in the study. Since this processing leads to an important gain in antimicrobial function and modification of host stimulatory effects by LL-37, activity of these enzymes, their expression, and balance with known skin serine protease inhibitors such as bikunin(28), elafin(29), anti-leukoprotease(30), and plasminogen activator inhibitor-2 (31), take on new found importance for understanding immune defense.

The gain in antimicrobial activity observed following processing of LL-37 to RK-31 and KS-30 was unexpected. Structural analysis of LL-37 has shown it is an antipathetic alpha-helical molecule that probably kills by ionic association with the membrane and subsequent disruption of the lipid bi-layer through formation of a toroidal pore. Change in the net charge by processing is an unlikely explanation for the increase in activity since the estimated PI of LL-37 is 10.4, identical to that of KS-30. Cleavage of the 6 amino acids at the amino terminus representing non-polar, polar and acidic residues leads to exposure of a basic residue that may contribute to the increase in activity. Previous analysis of the structurally distinct cathelicidin PR-39 has shown that N-terminal lysines are important

to activity and may function by facilitating initial ionic interaction with the anionic microbial surface (32). This explanation is likely incomplete since one of the largest gains in function seen by processing of LL-37 to shorter peptides was in its ability to kill wild-type *S.aureus*, an organism that has developed apparent resistance to cationic antimicrobial peptides such as beta defensins and LL-37 by modification of charge at the cell surface. Furthermore, structural modifications of defensins have major effects on chemotactic activity and lesser influence on antibacterial function (33), a phenomenon similar to that seen here with the loss of IL-8 stimulatory capacity. Additional high resolution structural studies of LL-37 and its shorter more active related peptides are necessary and may yield important new information relevant to the mechanism of action.

Understanding the role of antimicrobial peptides in mammalian immunity is complicated by many observations that these peptides also act on the host to stimulate a variety of important responses related to defense against injury. Cathelicidins were first found in mammalian skin due to their ability to increase fibroblast proteoglycan synthesis(34). Defensins and cathelicidins have both been shown to have chemotactic activity, possibly functioning through specific receptors such as CCR6 or FPRL-1 respectively(21, 35). Consistent with these prior observations, LL-37 was found in the present study to be a potent stimulus for IL-8 release from cultured keratinocytes. This effect was diminished upon processing to RK-31 or KS-30, and almost completely eliminated in the shortest KR-20 cathelicidin. It is not clear if topical LL-37 secreted into sweat would have a similar affect *in vivo* on epidermal keratinocytes when separated by the formidable barrier of the stratum corneum. However, the inhibition of pro-inflammatory functions by LL-37 in normal epithelia would be a beneficial mechanism to regulate unintended inflammation.

The present findings show post-secretory processing of LL-37 occurs at the skin surface. Generation of additional potent antimicrobial peptides suggests a model in which a single gene of the innate defense system can generate multiple differentially active products. Cathelicidins found in mammalian species such as the cow, pig, sheep, and horse are found as multiple copies encoding distinct C-terminal peptides. Conversely, in man and rodents, only a single cathelicidin gene product is known. Following initial processing of the pro-protein into the cathelin-like domain and LL-37, two

distinct defense molecules are activated; the cathelin-like domain possessing both antimicrobial activity and an ability to act as a protease inhibitor, and LL-37 having antimicrobial function combined with a range of host stimulatory capacities. The present findings suggest that in humans, further cathelicidin diversity is generated by post-secretory processing, tipping the balance of function towards antimicrobial action and away from affects on the host. Thus, the single human cathelicidin gene generates multiple products with a range of biological activities, each relevant to the local environment in which they are released.

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Figure Legends

Figure 1.

Soluble antimicrobial activity on human skin

Human sweat was concentrated 50X and separated by HPLC on C18. **a)** Absorbance profile at 214 nm for eluted material from 35% to 60% acetonitrile, inset: complete absorbance profile of eluted materials. **b)** Ability of material eluted in Fig 1a to inhibit growth of *S. aureus mprF* is shown as diameter of zone of *S. aureus mprF* inhibition. Several antimicrobial fractions were detected. Mass spectrometry identified 3 previously described antimicrobials; fractions labeled (1) and (3) are dermcidin and DCD-1L respectively (MW 4701, MW 4818, respectively) confirmed by N-terminal sequencing ((1): SSLLEKGLDGA, (3): SSLLE,). (2): LL-37 identified by mass spectrometry (MW 4493) and immunoblot. Data representative of single experiment repeated 5 times with separate sweat preparations.

Figure 2.

Serine protease in sweat enhances cathelicidin antimicrobial activity

Antibacterial activity evaluated by radial diffusion assay against *S. aureus mprF* after incubation of LL-37 (32uM) in sweat. **a)** Increase in the inhibition zone is seen when incubated in sweat but not 10 mM PBS or distilled water (DW). **b)** Diameters of inhibition zone after 6hr incubation at 37°C in (SWT buffer; sweat buffer salts alone), (PBS), (D.W.; distilled water), (SWT; sterile filtered human sweat at 37°C), (SWT at 4°C), (SWT with PI, with protease inhibitor cocktail). **c)** Action of specific protease inhibitors on gain of antimicrobial activity. Data show increase in inhibition zone at 37°C after 6hr compared to 0hr. Serine protease inhibitors AEBSF and Aprotinin were most effective. N.E.I. = neutrophil elastase inhibitor, L.E.I.= leukocyte elastase inhibitor. Data are triplicate determinations ±SEM from single experiment representative of 3.

Figure 3.

Purification of novel cathelicidin peptides generated from LL-37

a) Human sweat was separated by HPLC on C18. Absorbance profile at 214 nm is shown for eluted material from 30% to 60% acetonitrile. Crude is sweat prior to addition of LL-37, overlay plots show separate runs of sweat following addition of 32nmoles of LL-37 and incubation for 0 to 24 hr at 37°C. b) Antibacterial activity with radial diffusion assay against *S. aureus mprF* of fractions eluted from 24hr sample in a. Peaks with antimicrobial activity are labeled 1 and 2 and 3.

Figure 4.

Identification of novel cathelicidin peptides generated from LL-37

Following purification shown in Figure 3, major bioactive fractions were identified. **a)** peak (1) in Fig3a identified as KR-20 by mass spec. (MW 2468), and N-terminal sequence KRIVQRIKDVF, **b)** peak (2) detected 2 peptides; RK-31 and KS-30 (MW 3647, 3803, and RKSKEKIG, KSKEKIGK, respectively), **c)** Western blot analysis with anti-LL-37 was done on all fractions from Fig 3, shown are fractions eluting at acetonitrile concentrations 46-55. Peaks labeled (2) and (3) in Figure 3 were immunoreactive. **d)** peak (3) identified as LL-37 (MW 4493). Data from single experiment representative of 3.

Figure 5.

Processing of LL-37 enhances antimicrobial activity

The antimicrobial activity of cathelicidin peptides was evaluated by radial diffusion assay against **(a)** *S.aureus* , **(b)** *E.coli* , **(c)** solution assay against *C.albicans* and **(d)** Group A Streptococci . The antimicrobial activity of each peptide was evaluated in 10%TSB/10mM phosphate buffer with several NaCl concentraions against *E.coli* (e) and *S.aureus* (f). Data shown are representative of triplicate determinations.

Figure 6.

Processing of LL-37 decreases ability to stimulate keratinocyte IL-8

Cathelicidin peptides were added to culture of normal human keratinocytes at a final concentration of 3 or 10 μM then IL-8 release determined after 8 hr. All samples were endotoxin free by limulus assay. Data are mean ±SEM of triplicate determinations.

FIGURE 1

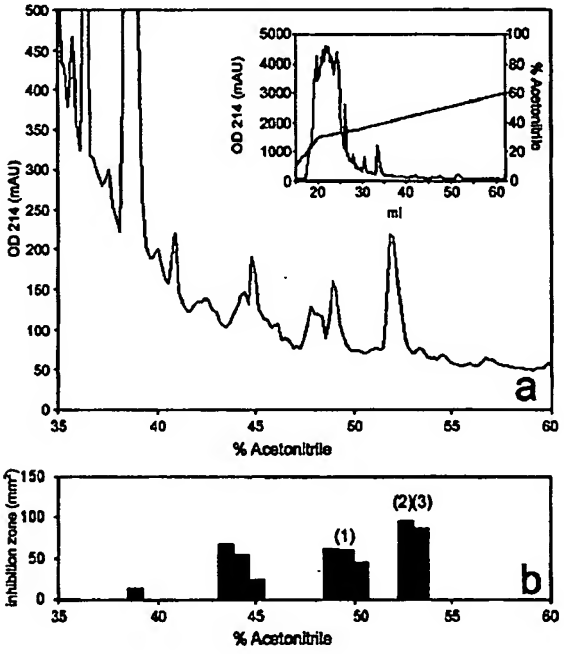

OD 214 (mAU)
% Acetonitrile
a
OD 214 (mAU)
% Acetonitrile
ml
Inhibition zone (mm²)
(1)
(2)(3)
b
% Acetonitrile

FIGURE 2

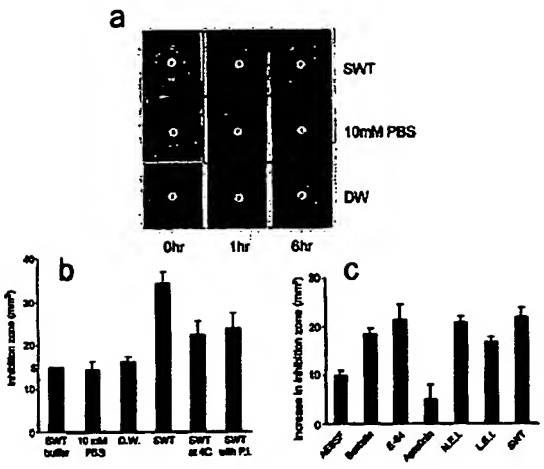


FIGURE 3

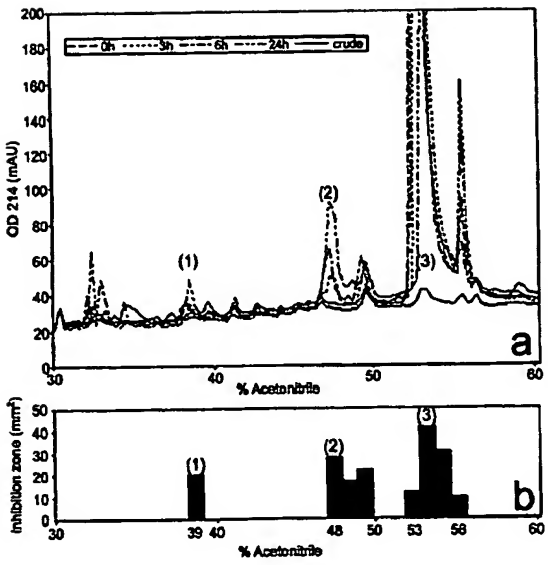

0h
3h
6h
24h
crude
OD 214 (mAU)
(1)
(2)
(3)
a
% Acetonitrile
Inhibition zone (mm²)
b
% Acetonitrile

FIGURE 4

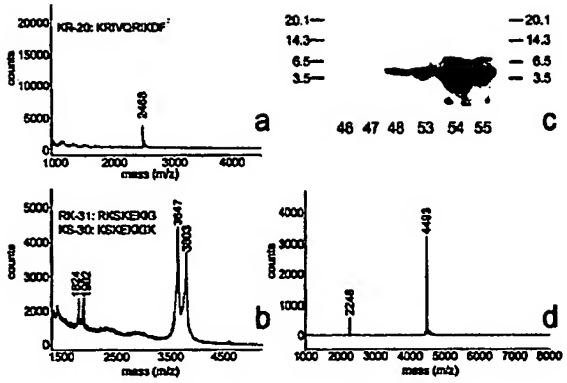

a
b
c
d
counts
mass (m/z)
2468
3647
3803
1824
1662
2248
4493
20.1
14.3
6.5
3.5
46 47 48 53 54 55

FIGURE 5

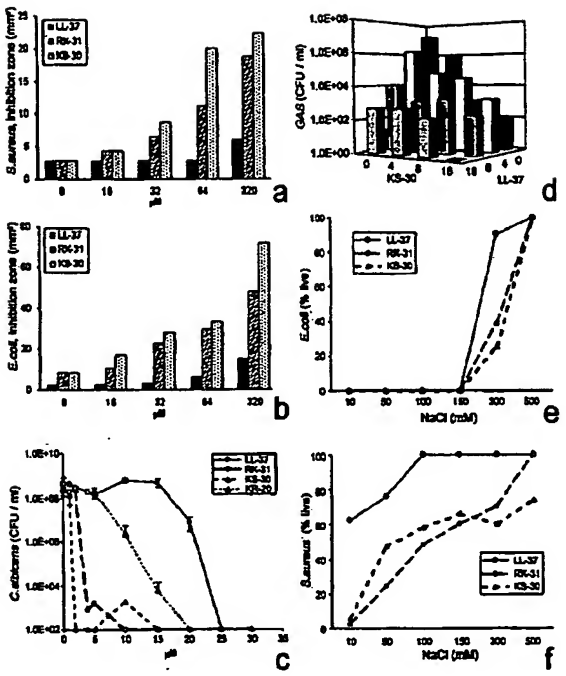

LL-37
RK-31
KS-30
S.aureus, inhibition zone (mm²)
a
GAS (CFU / ml)
d
E.coli, inhibition zone (mm²)
b
E.coli (% live)
NaCl (mM)
e
C.albicans (CFU / ml)
c
S.aureus (% live)
f

FIGURE 6

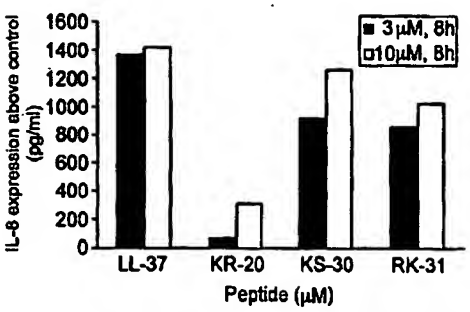
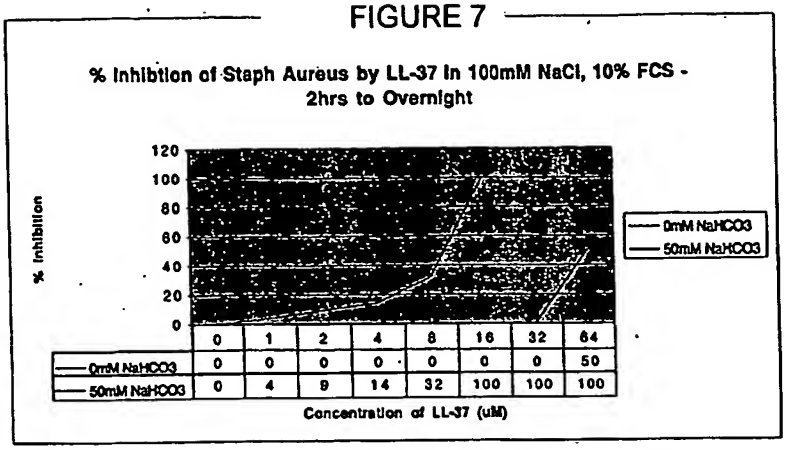
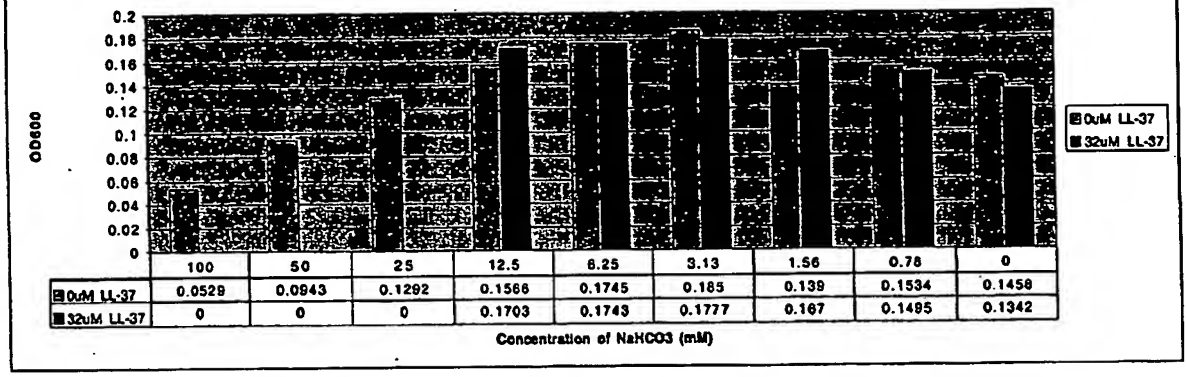

IL-8 expression above control (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
3μM, 8h
10μM, 8h
LL-37
KR-20
KS-30
RK-31
Peptide (μM)

FIGURE 7



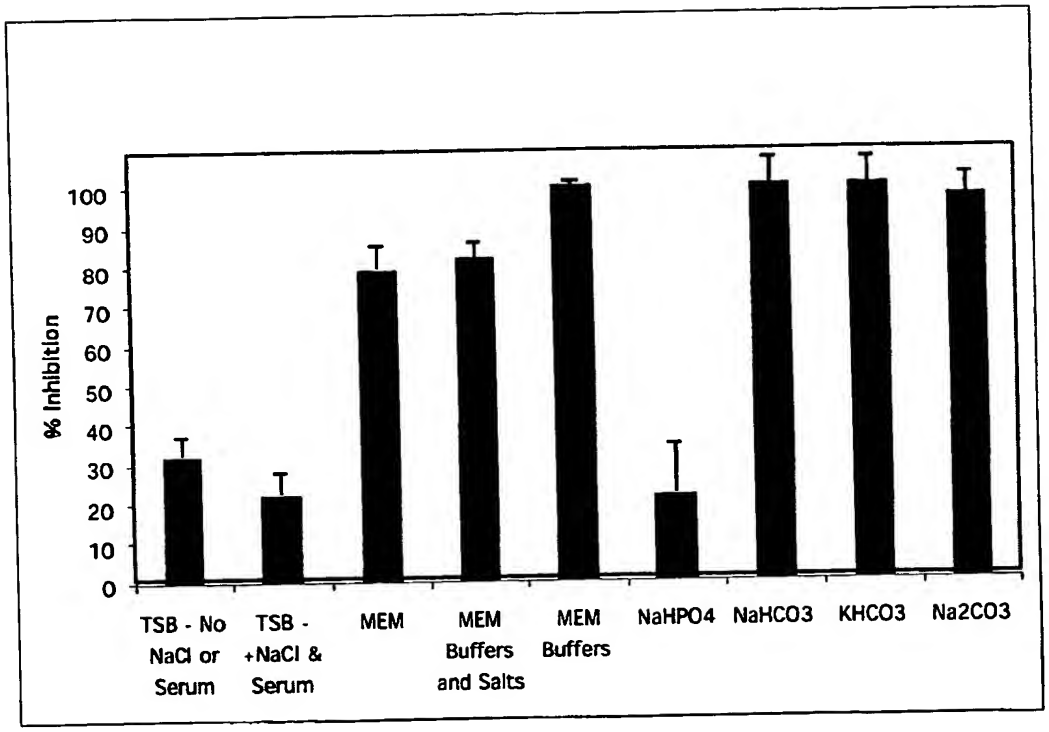
	0	1	2	4	8	16	32	64
0mM NaHCO3	0	0	0	0	0	0	0	50
50mM NaHCO3	0	4	9	14	32	100	100	100

FIGURE 8



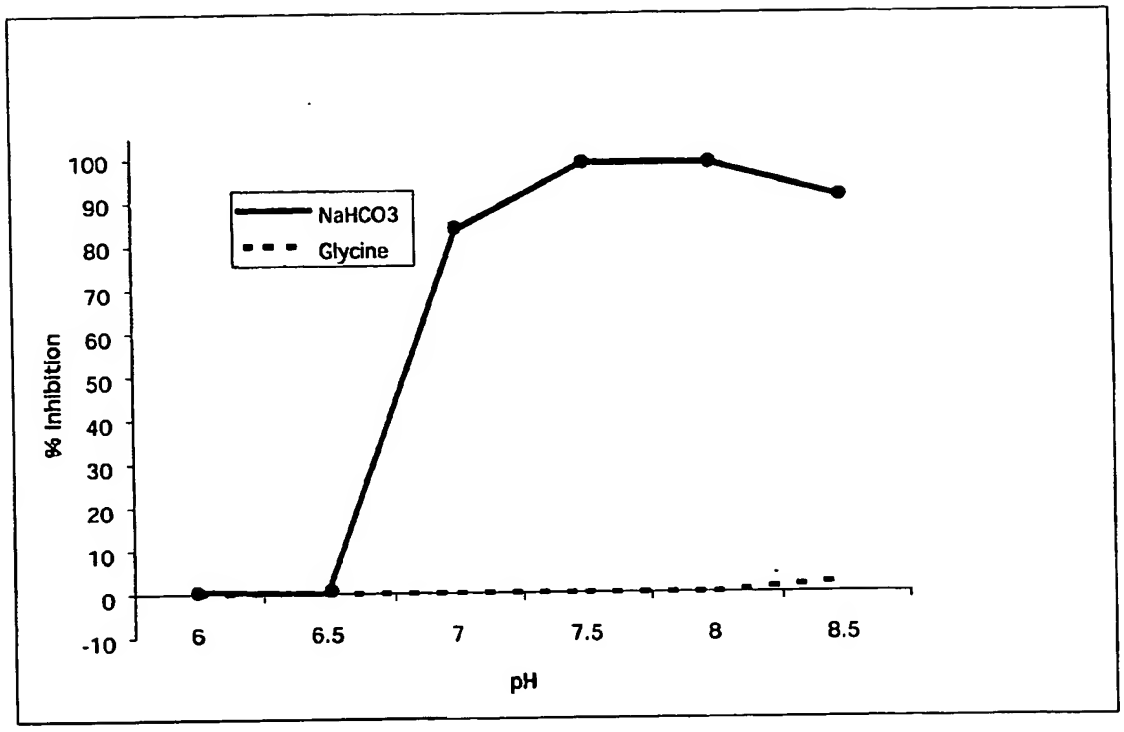
	100	50	25	12.5	6.25	3.13	1.56	0.78	0
0uM LL-37	0.0529	0.0943	0.1292	0.1566	0.1745	0.185	0.139	0.1534	0.1458
32uM LL-37	0	0	0	0.1703	0.1743	0.1777	0.167	0.1495	0.1342

FIGURE 9



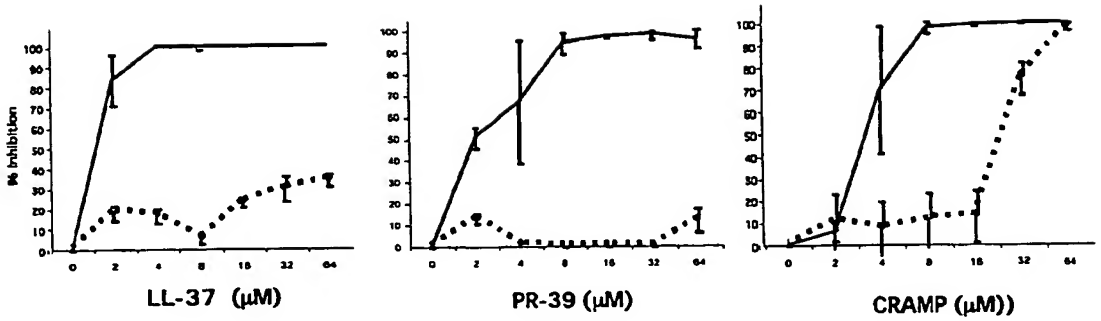
Staph aureus
32 uM LL-37
20% TSB
10% FCS
pH 7.4

FIGURE 10



Staph aureus
32 uM LL-37
20% TSB
150 mM NaCl
10% FCS

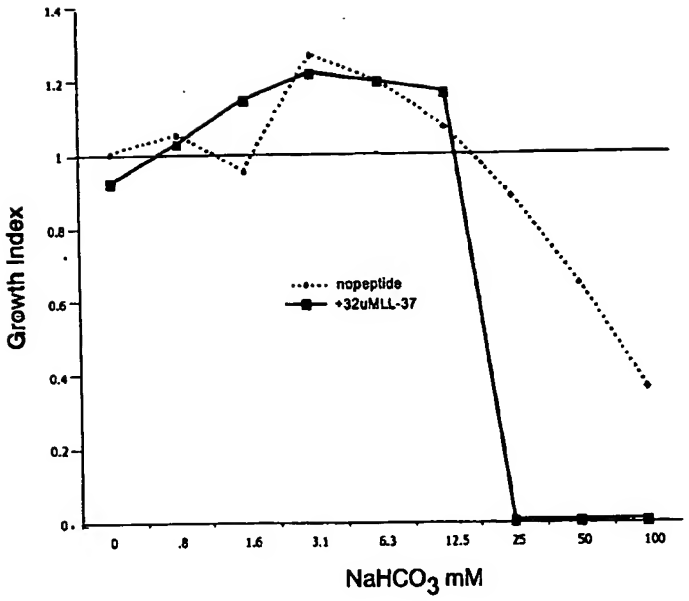
FIGURE 11



Staph aureus
20% TSB
NO NaCl
No FCS

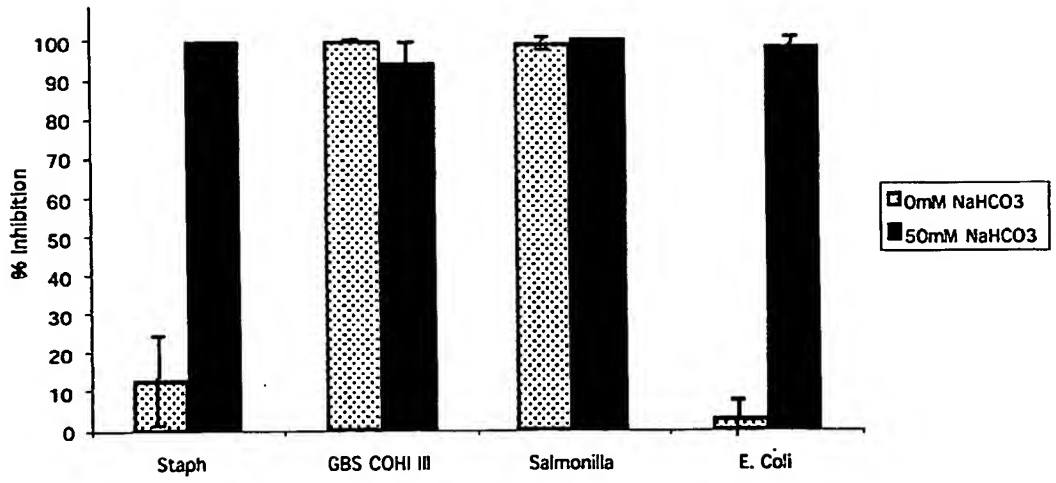
- - - 0mM NaHCO3
——— 50mM NaHCO3

FIGURE 12



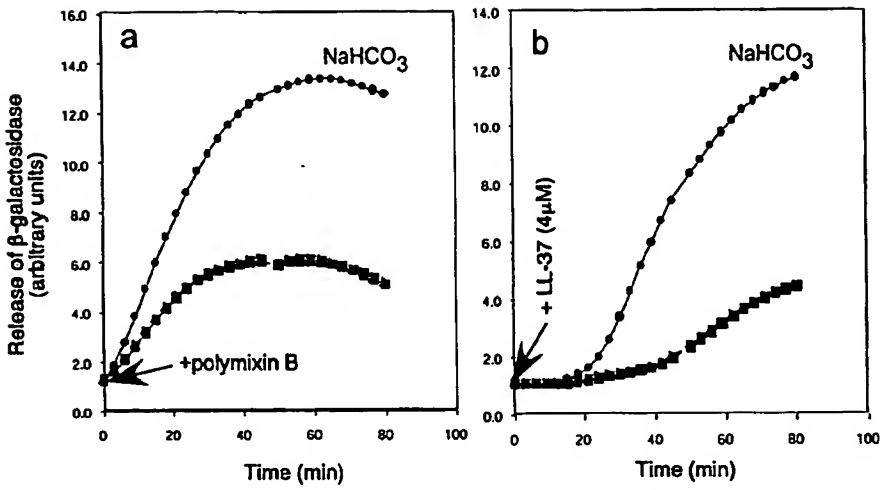
Staph aureus
pH 7.4
20% TSB
150 mM NaCl
10% FCS

FIGURE 13



Cramp at 16 uM
20% TSB
no NaCl or FCS
pH 7.4

FIGURE 14



e. coli inner membrane permeability
no NaCL, FCS
pH 7.4
data are OD 420 with antibiotic/no antibiotic

FIGURE 15

Effect of EF on the Inhibitory Activity of Various Antimicrobial Peptides on Staph Aureus
(+150mM NaCl and 10% FCS)

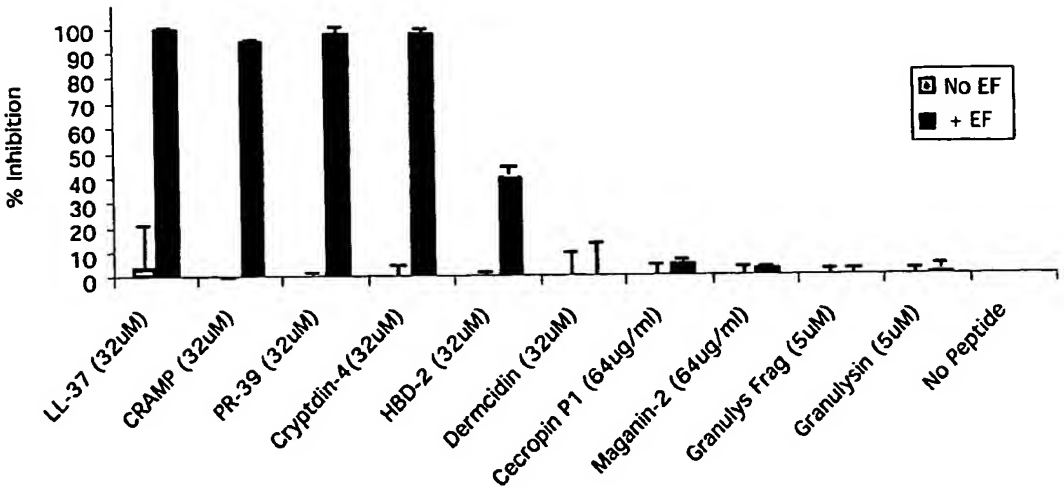


FIGURE 16

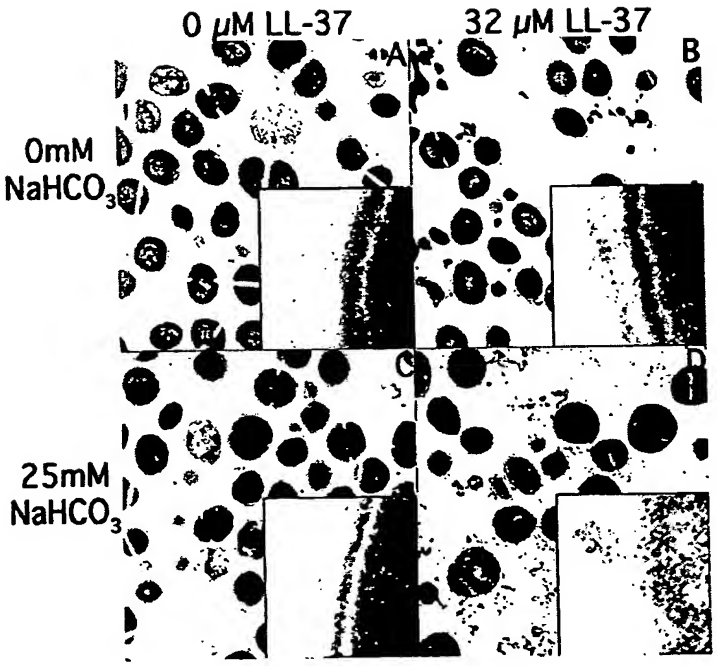
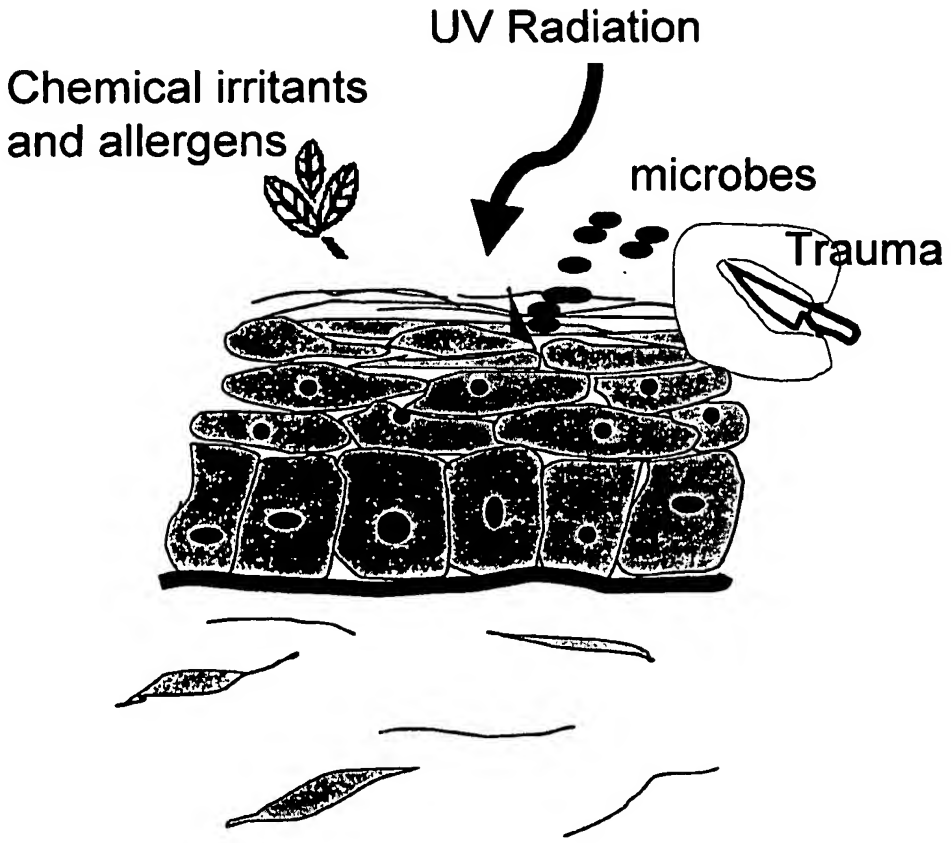


FIGURE 17 – 40pg

Antimicrobial Peptides and Innate defense of Skin

Richard L. Gallo, M.D.,Ph.D.
University of California, San Diego

Skin as a simple Barrier



Immune Defense System

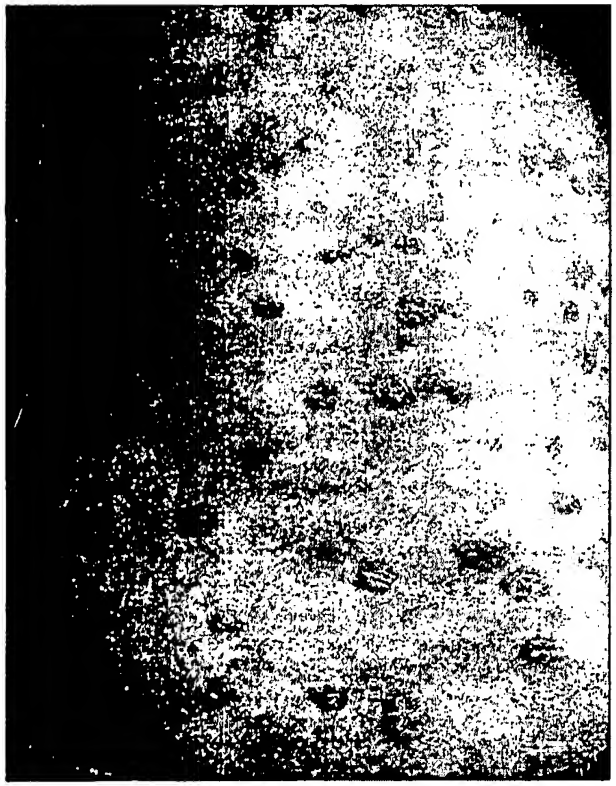
1. Physical Barrier
2. Chemical (NO, H_2O_2)
3. Inflammatory
   - Cytokines
   - Chemokines
   - Neuropeptides
   - Eicosanoids
4. Cellular
   - Neutrophils
   - Macrophages
   - Natural Killer
5. Adaptive
   - Dendritic cells
   - T and B cells

How do we explain common skin diseases with this model?

Onychomycosis:

Nail Plate infected
No opportunity for direct cell-mediated defense

How do we explain common skin diseases with this model?

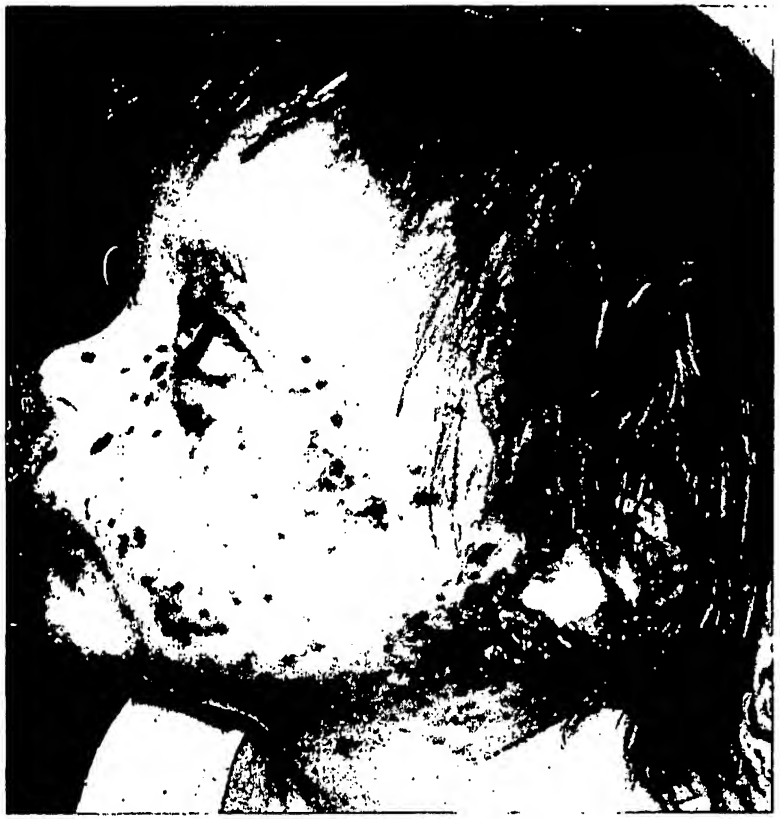


Psoriasis:

Triggered by:
stress, trauma, infection

Patients have fewer infections

How do we explain common skin diseases with this model?



Atopic Dermatitis, Eczema Herpeticum:

Increased inflammation
but
Increased infections

How do we explain common skin diseases with this model?

Rosacea:

Triggered by:
vasodilitation, bacteria

Patients have chronic infection
without apparent immune deficit

The answer may be to understand “innate immunity”

Innate Immunity

- non-adaptive (no need to learn)
- non-clonal (not dependent on cell proliferation)
- rapid response to danger (injury or infection)
- evolutionarily conserved (insects, plants)
- adapted by mammals (acts on cellular immunity)

Insects and lower organisms depend on innate immunity

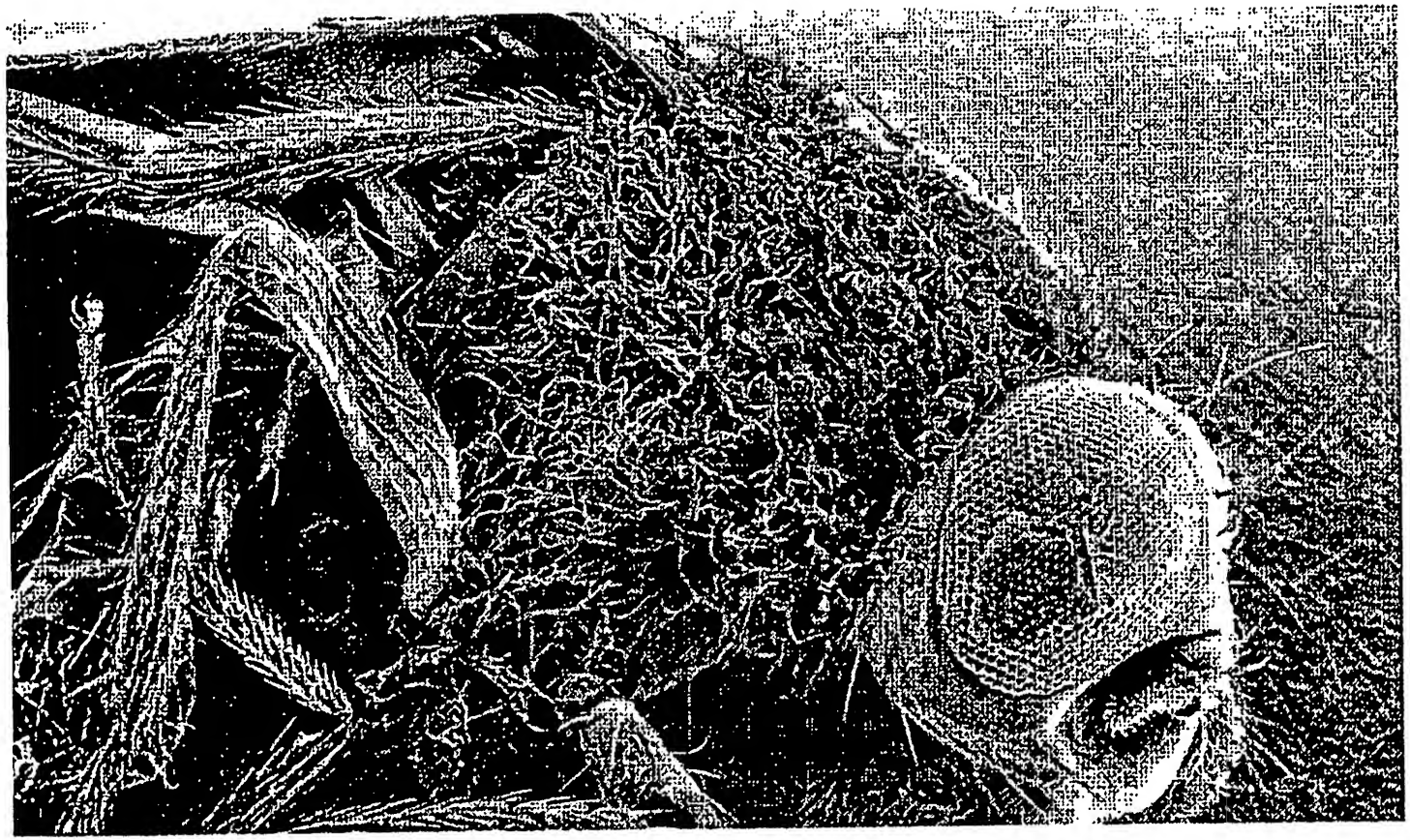


Figure 5. Germinating Hyphes of A. fumigatus on a Dead Drosophila

Scanning electron micrograph of a Drosophila adult that succumbed to infection by A. fumigatus and is covered with germinating hyphae (200× magnification).

Cell 1996 86(6):973-83

Innate Immunity in 2003

Found in all multicellular organisms

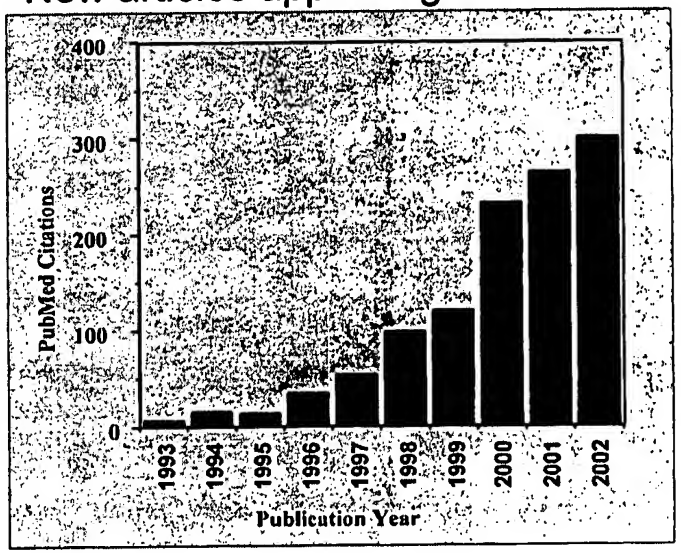
- Dependent on specific pattern recognition
- Multiple effector mechanisms

Close interactions with adaptive immunity

- Increasing interest

Essential element in skin defense

New articles appearing in PubMed



Multiple Effector Elements of Innate Immunity

Type	Examples
Inorganic molecules	HCl, H_2O_2, nitric oxide
Simple Organics	fatty acids, lipids
Binding Proteins	Mannose binding protein
Cytokines	IL-10, IL-12, TNF-alpha
Cells	Macs, Polys, NK
Proteins	BPI, lysozyme,

Antimicrobial Peptides

Many Peptides with Antimicrobial Action

Bombinins
- amphibians

Cecropins
- insects
- mammals

Dermaseptins
- amphibians

Magainins
- amphibians

Defensins
- plants
- insects
- mammals → HBD-2, -3

Cathelicidins
- mammals → LL-37

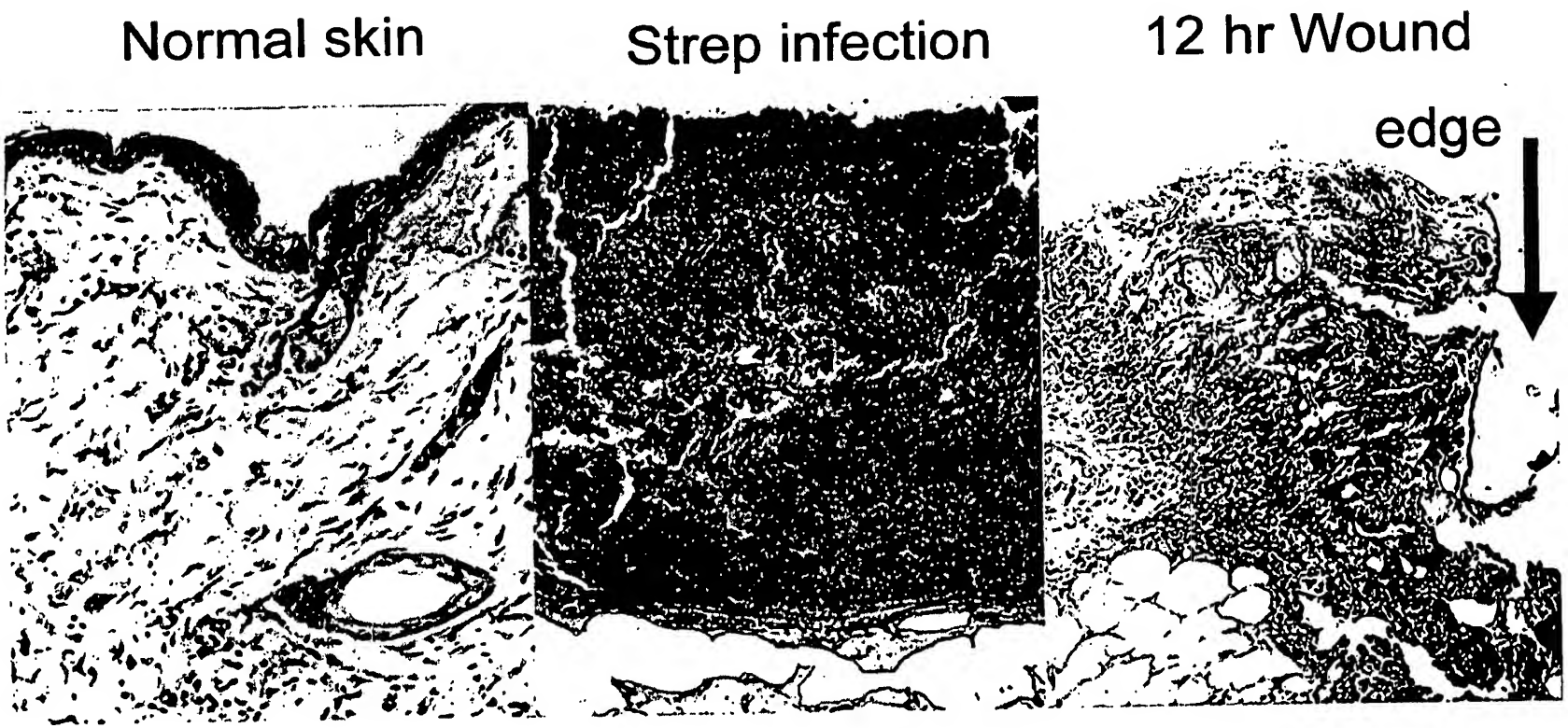
Granulysin

Human skin

Over 800+ peptides known

For review see Nature (2002) 415:389-395 and others

In Skin, Injury or Infection Triggers Antimicrobial Peptide Expression

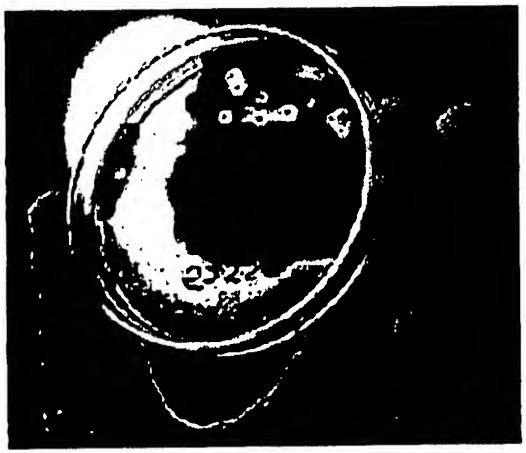


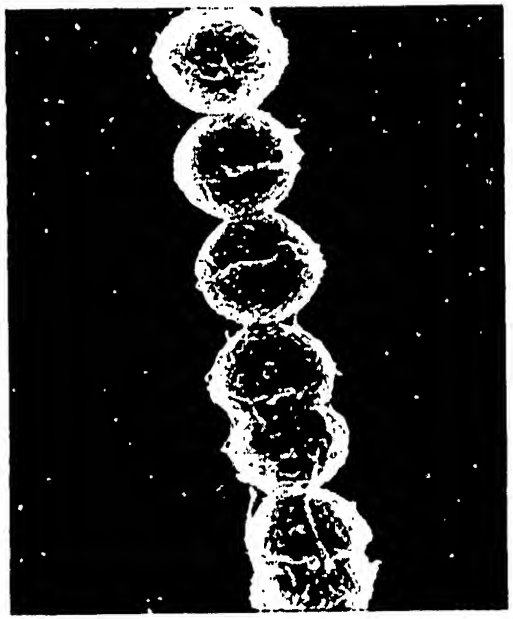
Brown staining= cathelicidin peptide immunoreactivity

J Invest Dermatol (2001) 117:91-97

Is an increase in antimicrobial peptides important?

Group A Streptococcus (*S. pyogenes*)





Sensitive to Cathelicidins

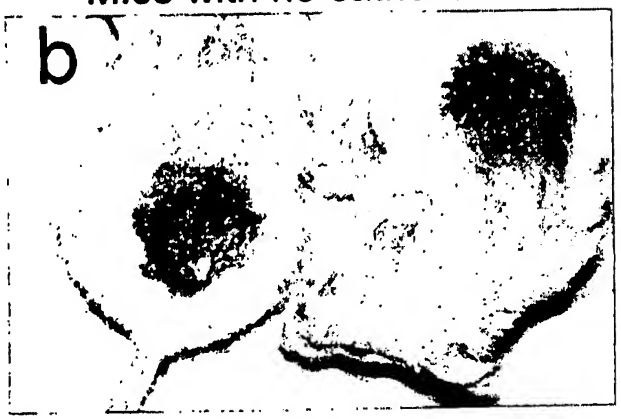
CRAMP: MIC = 2 - 8 μM

LL-37: MIC = 8 - 24 μM

In mice, cathelicidin knock-outs are more susceptible to Invasive Group A *Streptococcus*

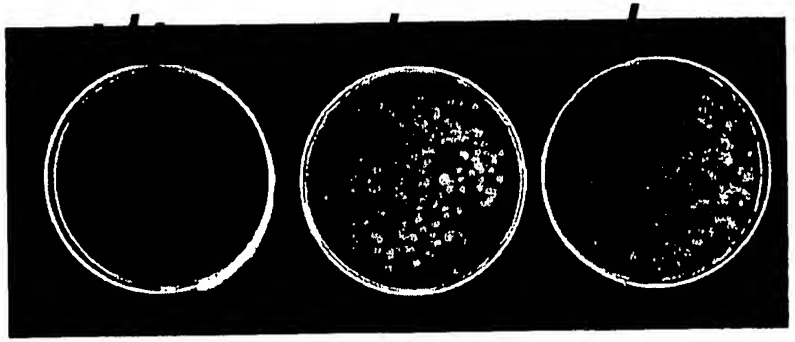
Mice with cathelicidin

Mice with no cathelicidin



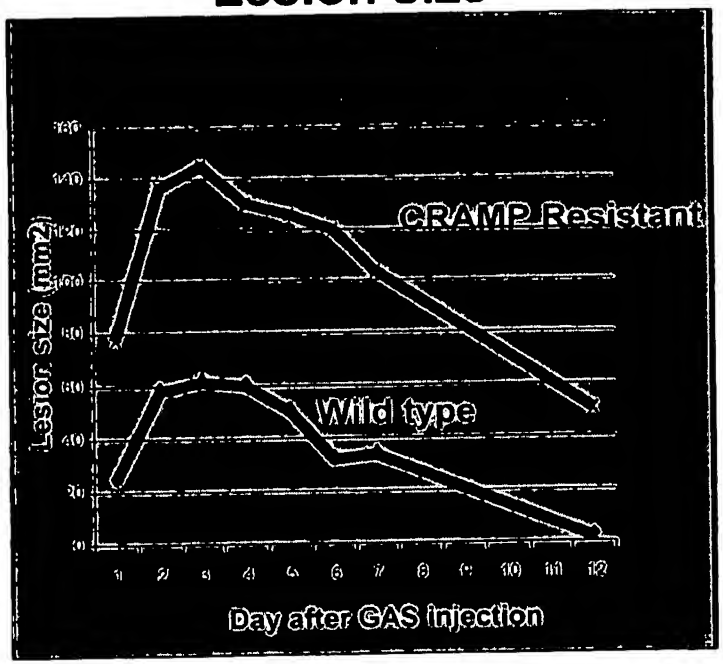
day 7

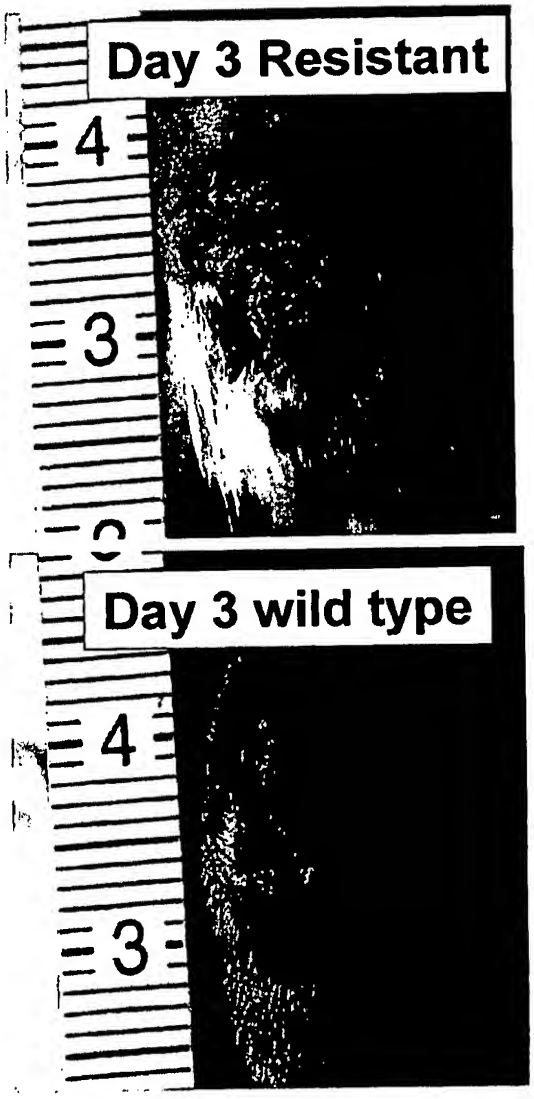
Lesion culture at 7 day



Nature, (2001) 414:454

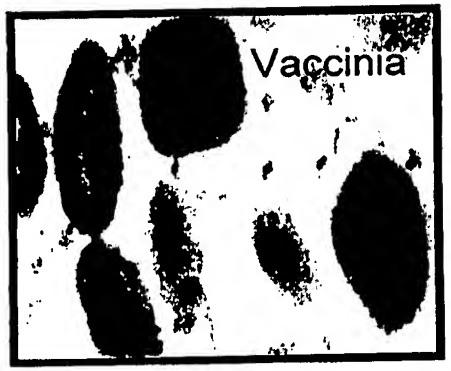
Bacteria (GAS) that are cathelicidin resistant are more pathogenic



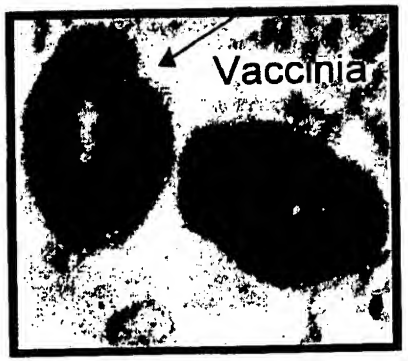


Nature, (2001) 414:454

Cathelicidins also provide defense against viral infection



No LL-37



LL-37 5 μM

No Lesion 15/16 Control Mice

LL-37 (μM)	Altered virions/ virion number (%)	
0	1/23	(4%)
5	19/28	(67%)
25	27/30	(90%)

Vesicular Lesion 4/6 CRAMP -/-

Conclusion:

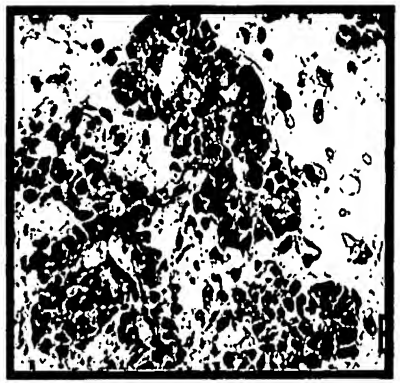
The presence of antimicrobial peptides is essential
for normal defense against certain bacterial and viral skin infections

How are the cathelicidin antimicrobial peptides used by the skin?

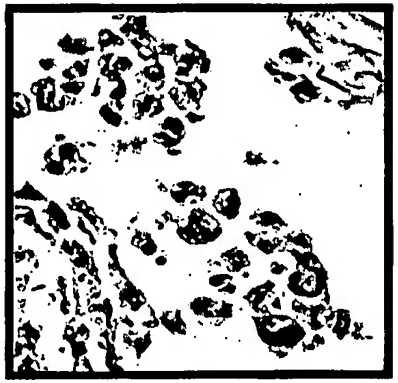
Cathelicidins are expressed at the interface

SKIN

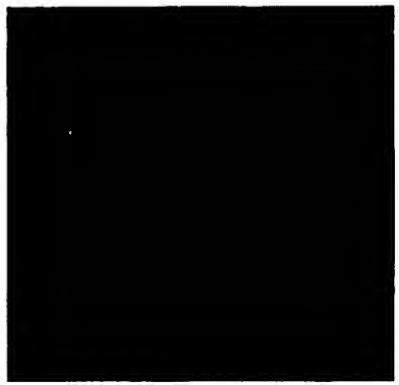
COLON



SALIVARY GLAND



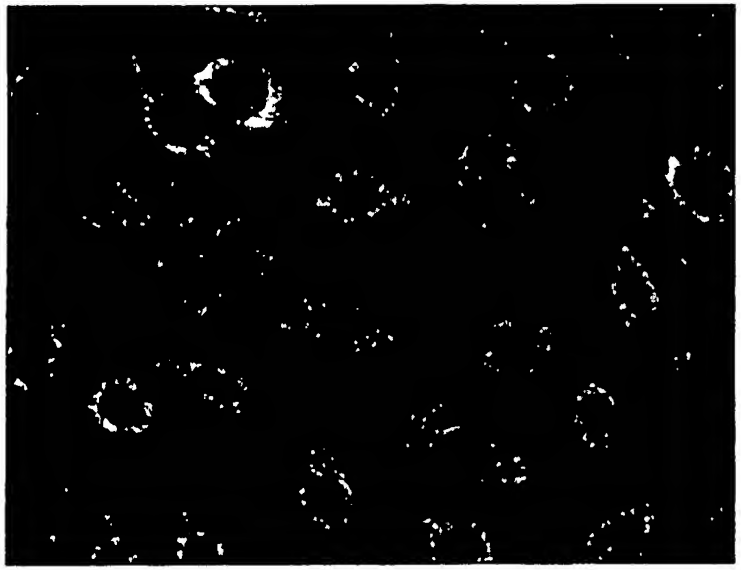
NEUTROPHIL



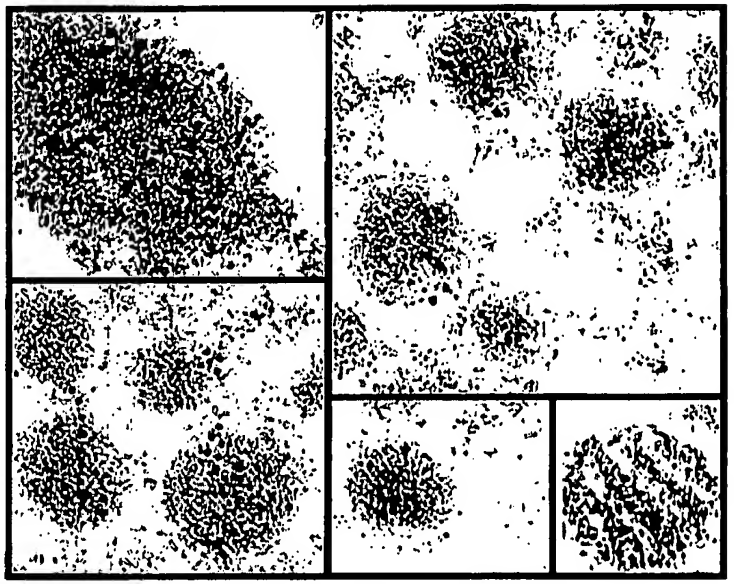
MAST CELL

CATHS ARE STORED IN KERATINOCTYE GRANULES

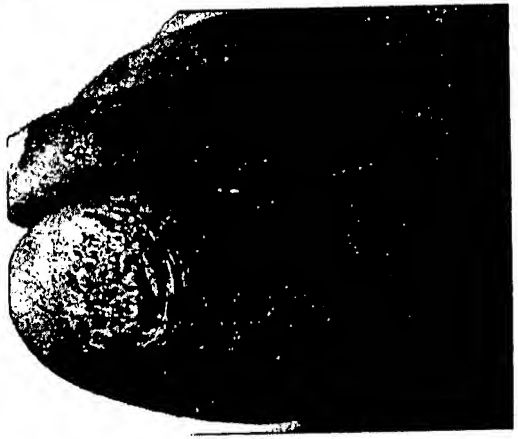
FITC Anti-LL37



Anti-LL37 immuno gold EM

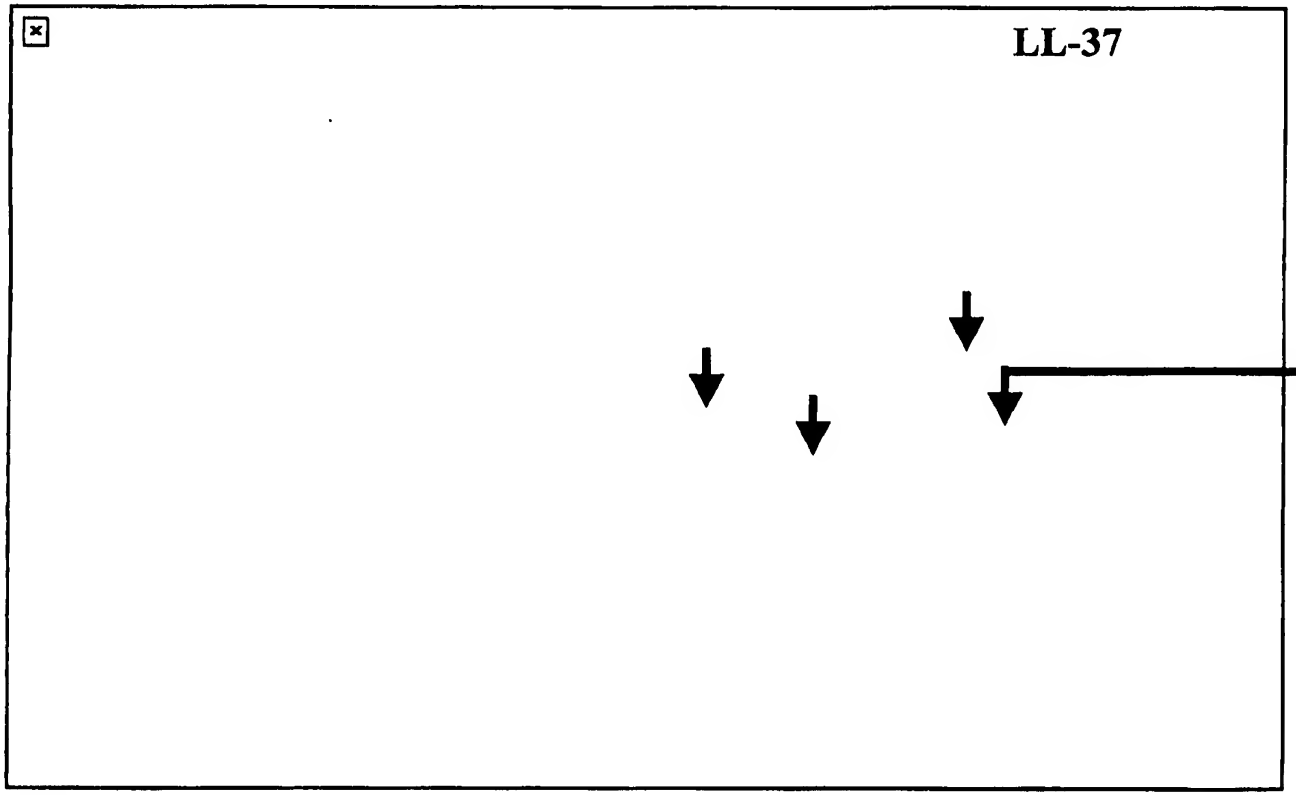


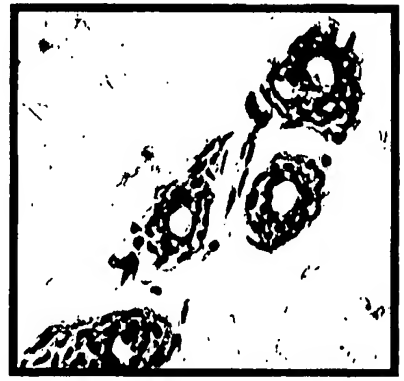
Caths are expressed in the nail apparatus



Is this an answer to normal nail defense?

Cathelicidin peptides discovered in sweat with increased activity





Eccrine gland and duct stain for cathelicidin

New bioactive forms

Kill bacteria better after processed by enzyme in sweat

Work of Dr. M. Murakami, now a Dermatology resident at Asahikawa

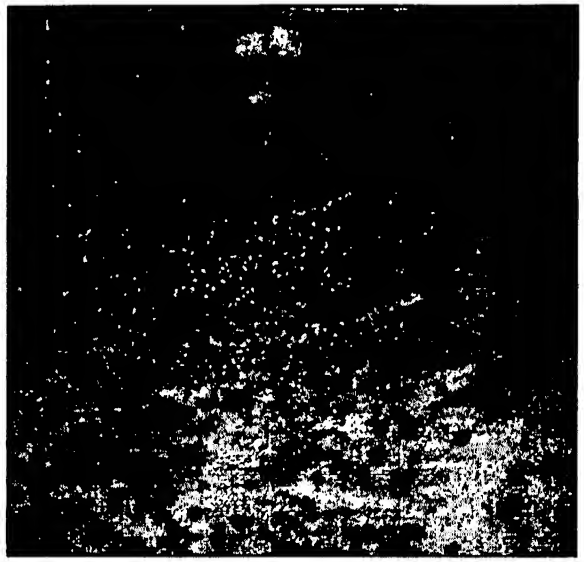
Does this correlate with human disease?

Atopic patients are susceptible to infection and have inflammation.
What is their expression of antimicrobial peptides?

Impetigo

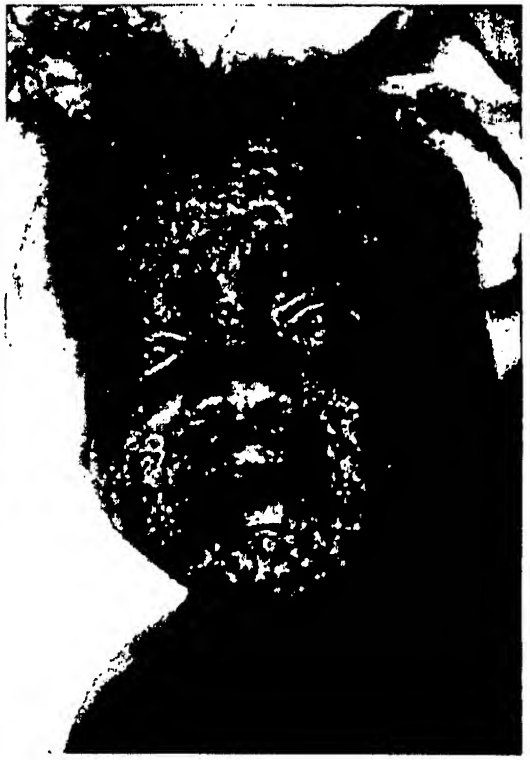
Staph
Strep

Eczema Herpeticum



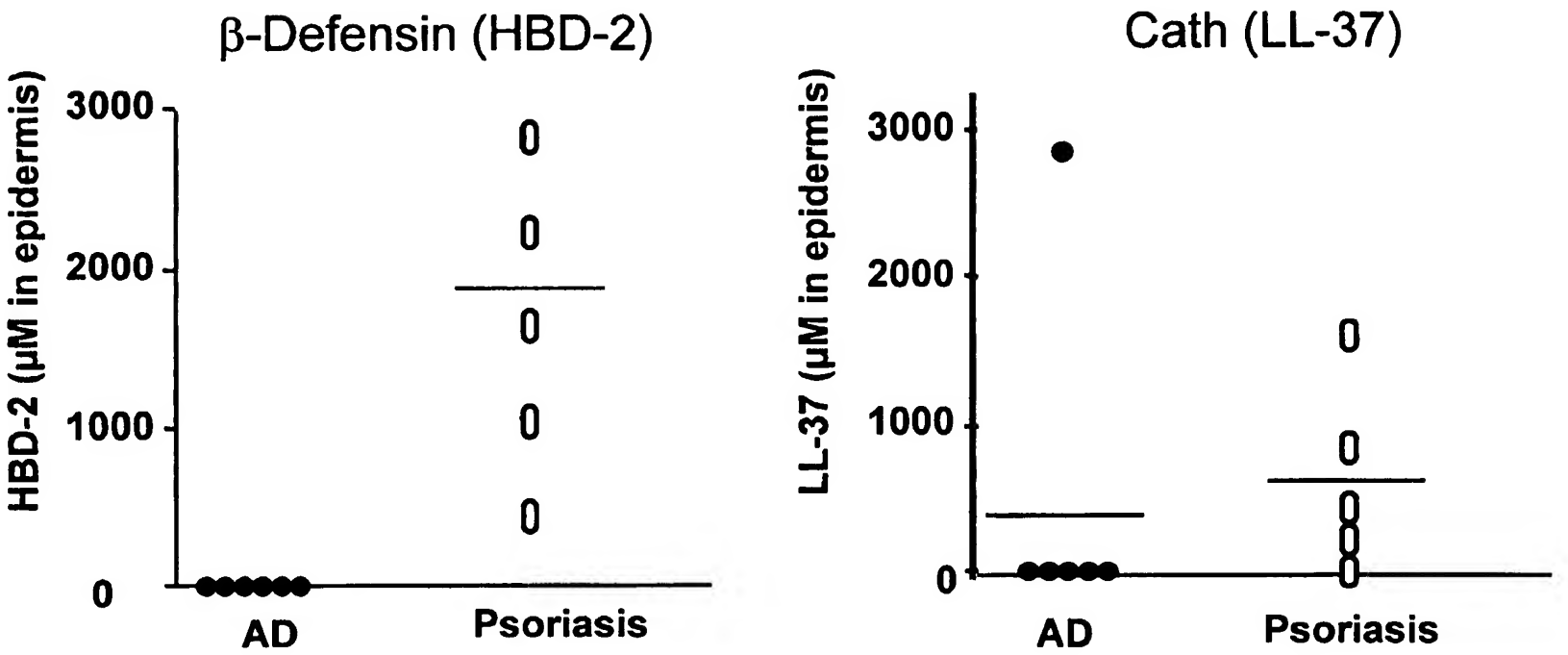
HSV, VZV

Eczema Vaccinatum



Cow pox

Compared to psoriasis, atopic patients do not respond to inflammation with a increase in antimicrobial peptides



Ong et al. (2002) N Engl J Med 347:1151-60

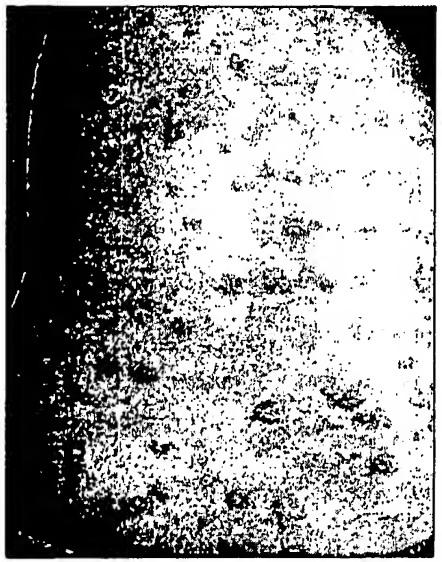
Work of Dr. T. Ohtake, now faculty in Medicine at Asahikawa

Conclusions

Cathelicidins are produced by several cells in the skin.
A lack of normal production correlates with disease

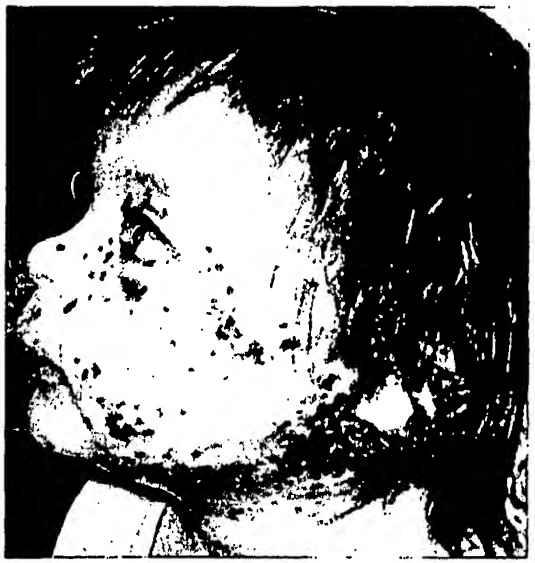
Psoriasis

Overproduction ➡ Protection



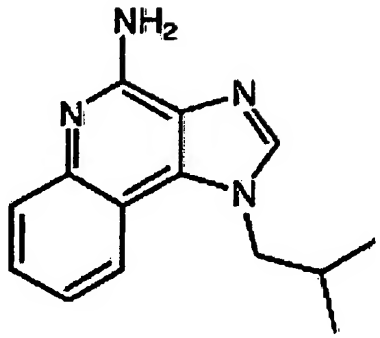
Atopic Dermatitis

Underproduction ➡ Infection

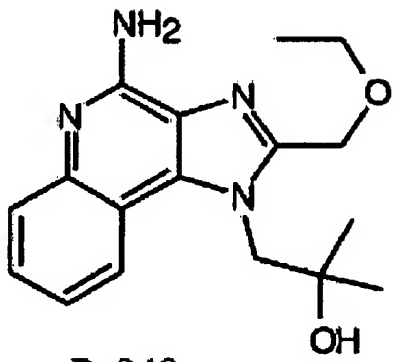


Can our increased understanding of innate immunity and antimicrobial peptides be used for therapy?

Imiquimod:



Imiquimod



R-848

Stimulus of TLR-7 provides useful pro-inflammatory tool.

Effective for:
Common warts, condylomata, molluscum,
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Antimicrobial peptides have been successfully used in preliminary animal models

Protection of burn wounds against infection
(2001) Crit Care Med 29:1431

Peritonitis
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Skin wounds
(2001) Blood 97:297

Cardiac angiogenesis
(2000) Nat Med 6:49

Lung Infection
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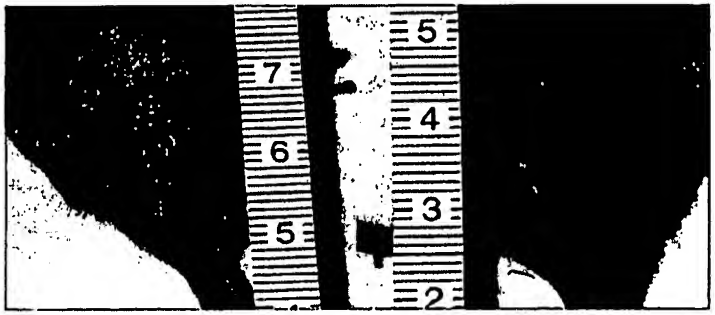
Antimicrobial gene therapy is effective in mice

PR-39 expression in wound lesion

anti-PR-39 pre-immune



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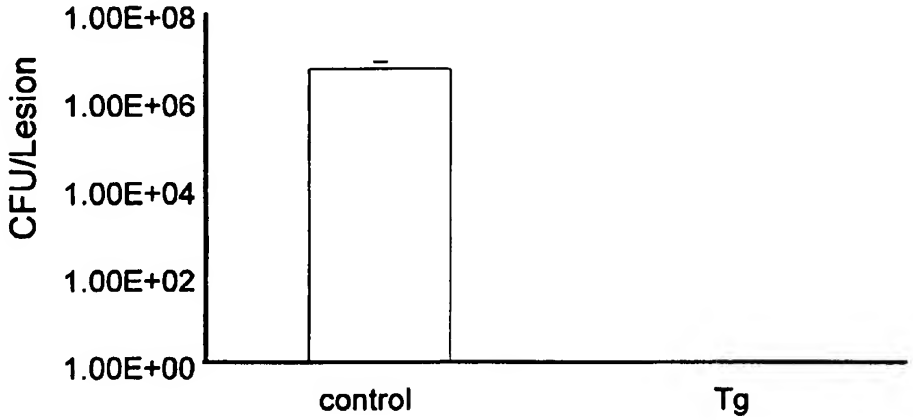
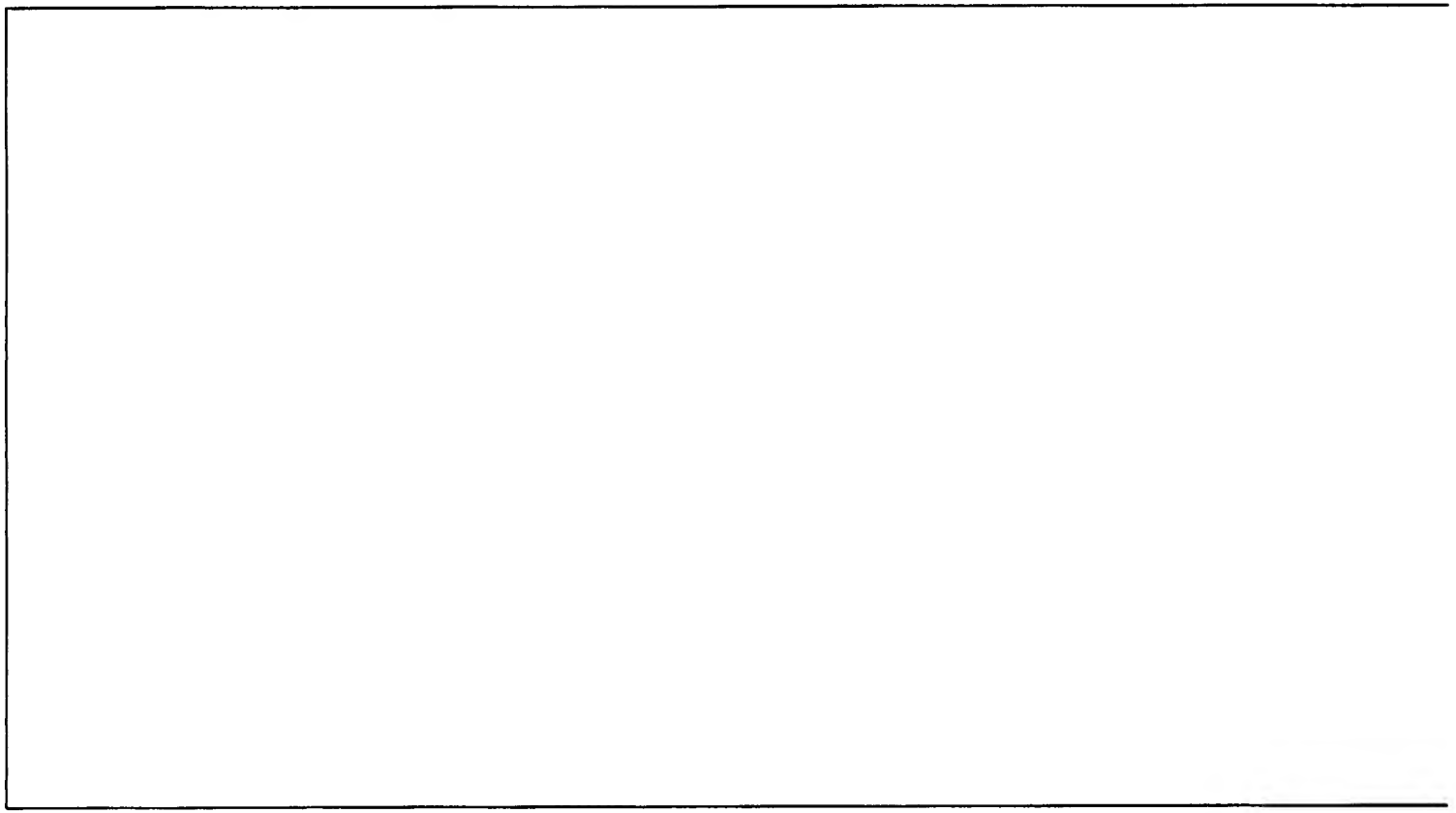
Live bacteria in lesion (day6)

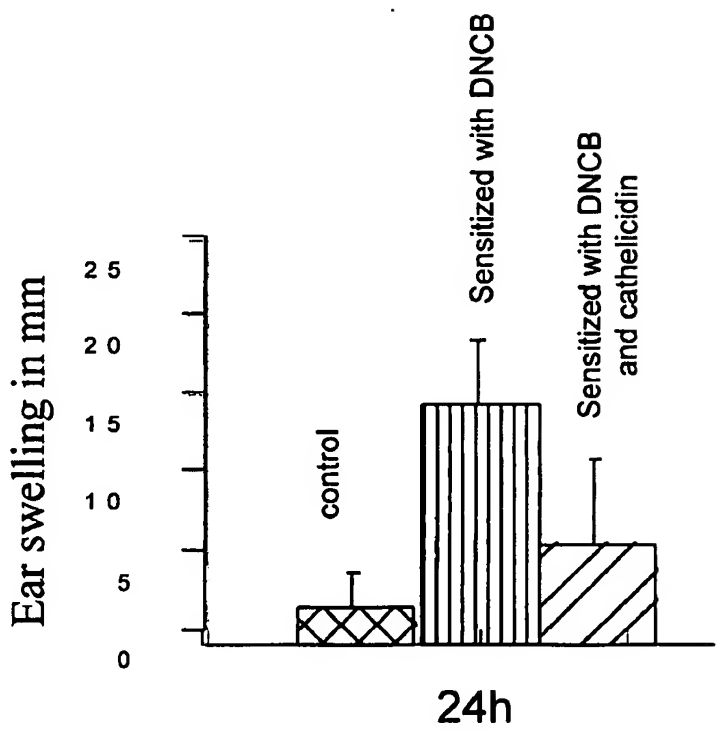


Human trials are underway

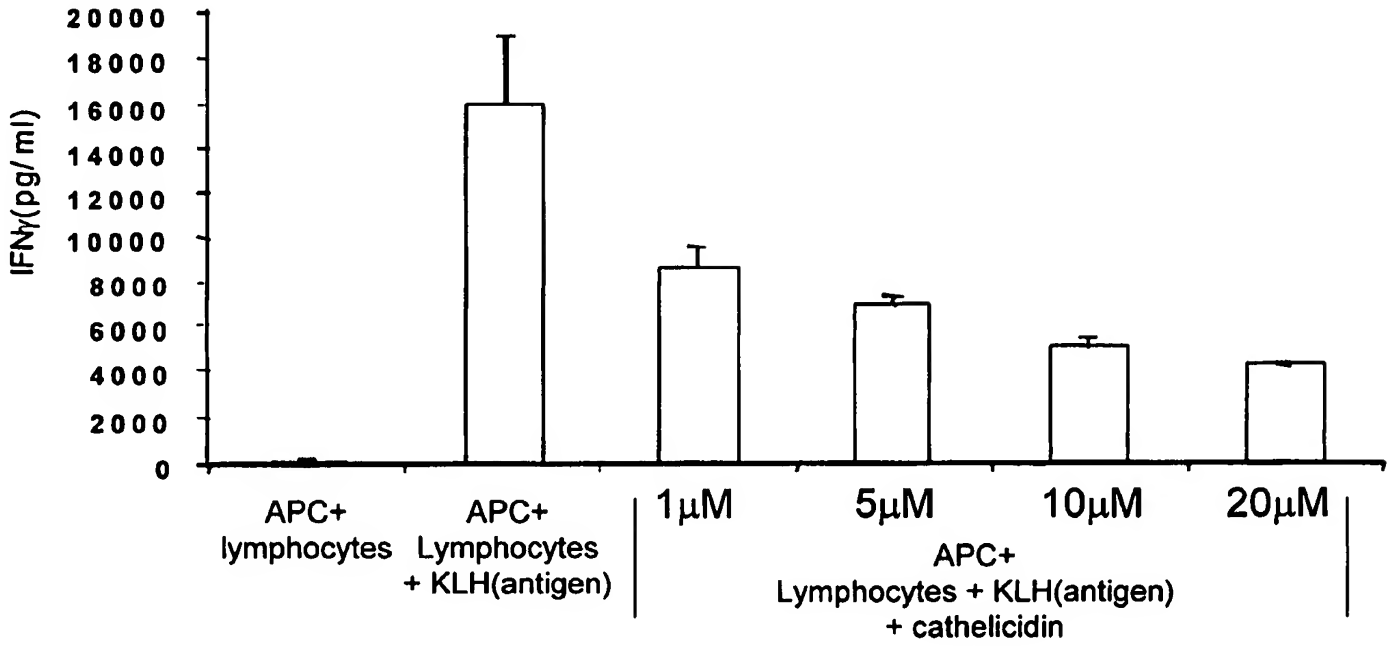


However,
Antimicrobial Peptides do more than kill microbes!

Cathelicidin blocks allergic contact dermatitis in mice



Cathelicidin blocks antigen presentation



But chronic administration of cathelicidin is proinflammatory

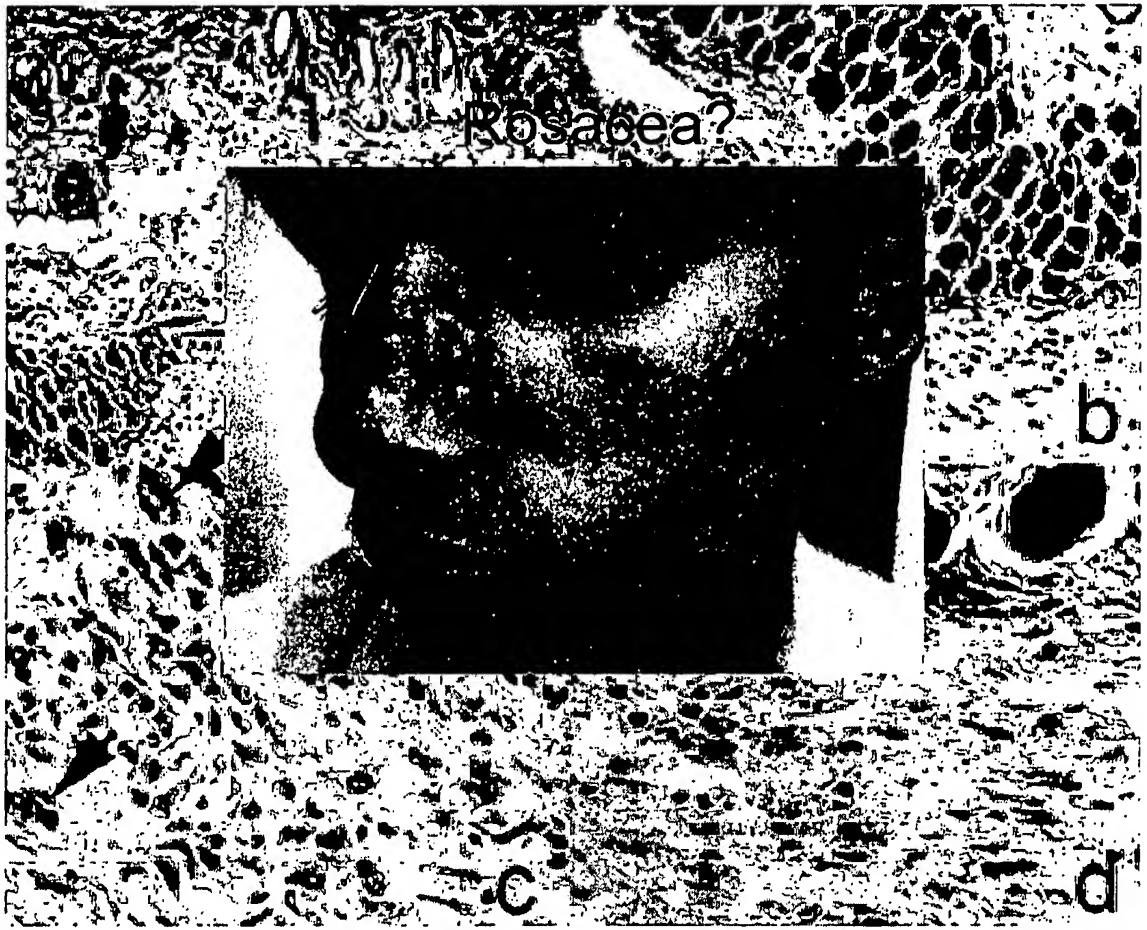
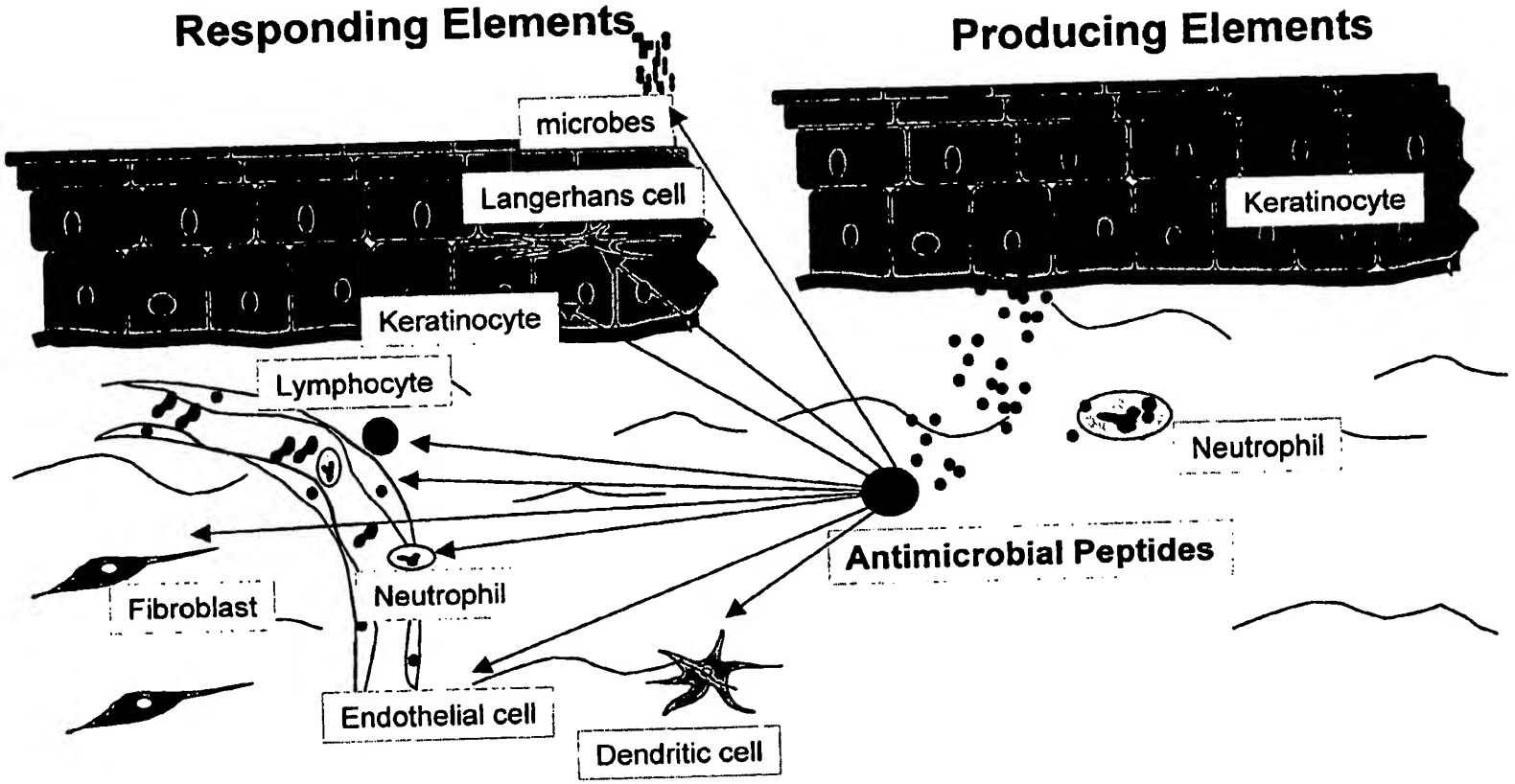


Figure 5

- Antimicrobial peptides are important to innate immune defense
- Defects in innate immunity may explain certain skin diseases
- Action of antimicrobial peptides are part of the whole immune system

SUMMARY:
Antimicrobial Peptides in the Skin



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Cathy Wong
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UCSD
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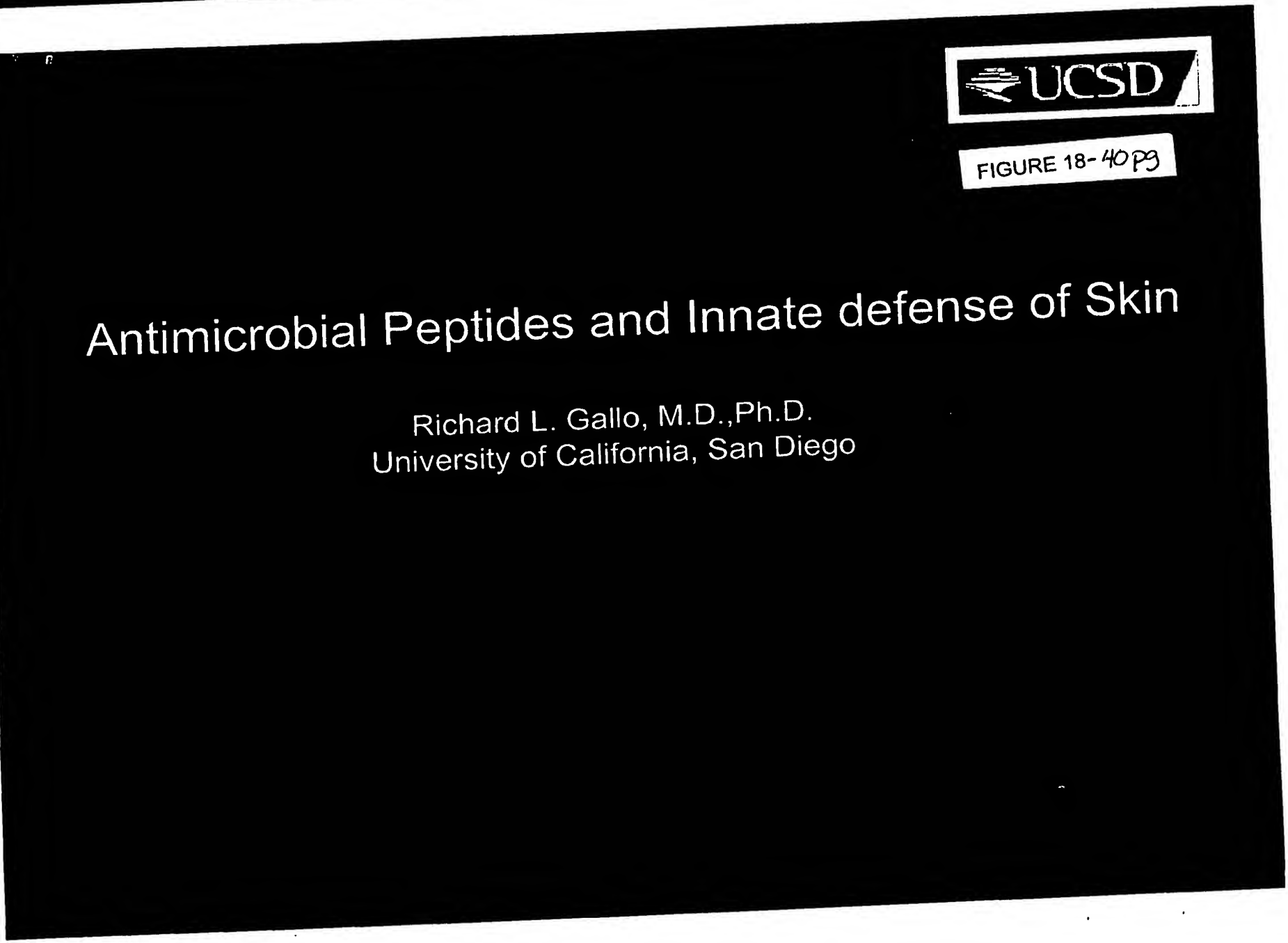
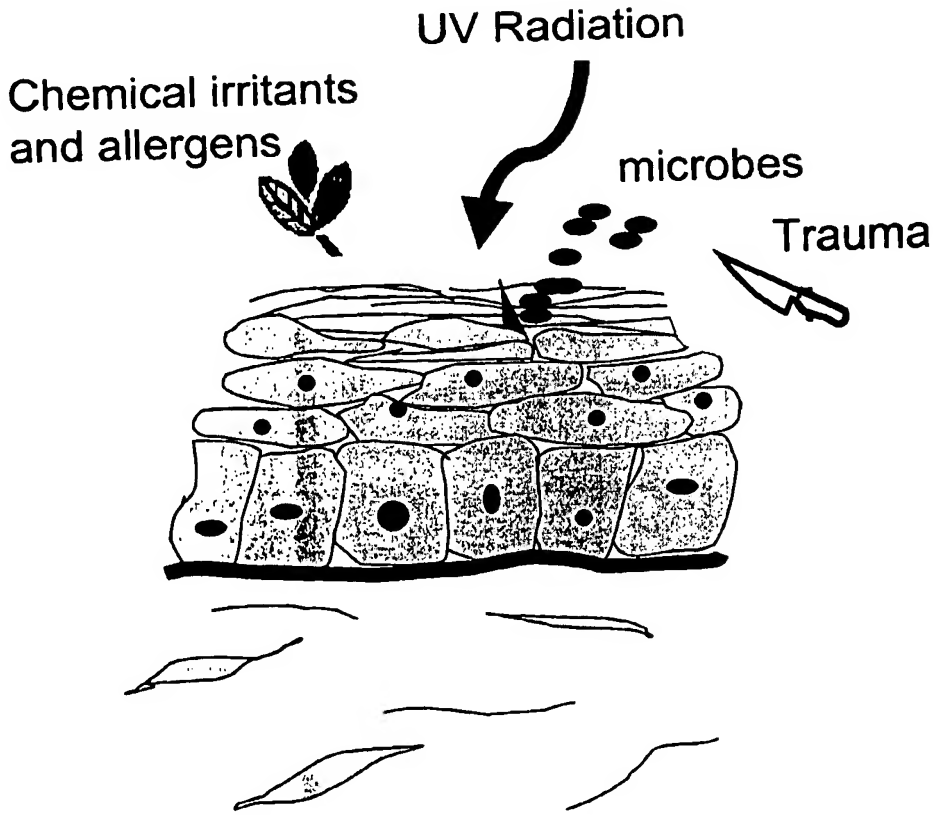

UCSD
FIGURE 18-40 pg
Antimicrobial Peptides and Innate defense of Skin
Richard L. Gallo, M.D.,Ph.D.
University of California, San Diego

Skin as a simple Barrier



Immune Defense System

- Physical Barrier
- Chemical (NO, H_2O_2)
- Inflammatory
  - Cytokines
  - Chemokines
  - Neuropeptides
  - Eicosanoids
- Cellular
  - Neutrophils
  - Macrophages
  - Natural Killer

5. Adaptive
   - Dendritic cells
   - T and B cells

How do we explain common skin diseases with this model?

Onychomycosis:

Nail Plate infected
No opportunity for direct cell-mediated defense

How do we explain common skin diseases with this model?

Psoriasis:

Triggered by:
stress, trauma, infection

Patients have fewer infections

How do we explain common skin diseases with this model?

Atopic Dermatitis, Eczema Herpeticum:

Increased inflammation
but
Increased infections

How do we explain common skin diseases with this model?

Rosacea:

Triggered by:
vasodilitation, bacteria

Patients have chronic infection
without apparent immune deficit

The answer may be to understand “innate immunity”

Innate Immunity

- non-adaptive (no need to learn)
- non-clonal (not dependent on cell proliferation)
- rapid response to danger (injury or infection)
- evolutionarily conserved (insects, plants)
- adapted by mammals (acts on cellular immunity)

Insects and lower organisms depend on innate immunity

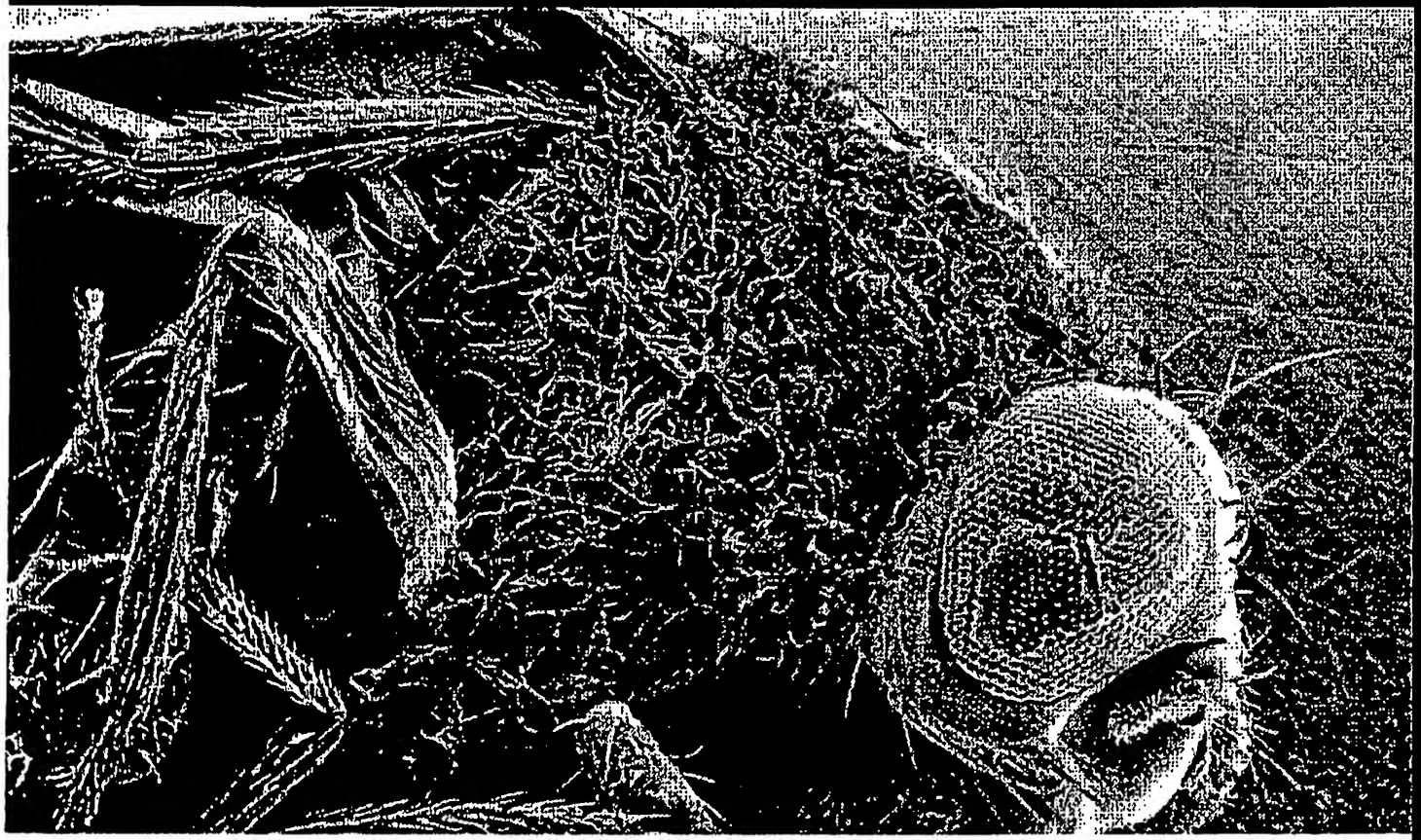


Figure 5. Germinating Hyphes of A. fumigatus on a Dead Drosophila

Scanning electron micrograph of a Drosophila adult that succumbed to infection by A. fumigatus and is covered with germinating hyphae (200× magnification).

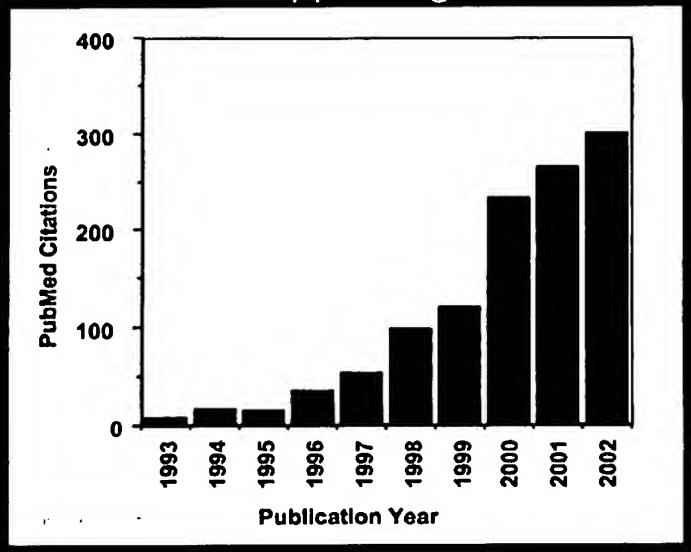
Cell 1996 86(6):973-83

Innate Immunity in 2003

- Found in all multicellular organisms
- Dependent on specific pattern recognition
- Multiple effector mechanisms
- Close interactions with adaptive immunity
- Increasing interest

Essential element in skin defense

New articles appearing in PubMed



Multiple Effector Elements of Innate Immunity

Type	Examples
Inorganic molecules	HCl, H_2O_2, nitric oxide
Simple Organics	fatty acids, lipids
Binding Proteins	Mannose binding protein
Cytokines	IL-10, IL-12, TNF-alpha
Cells	Macs, Polys, NK
Proteins	BPI, lysozyme,

Antimicrobial Peptides

Many Peptides with Antimicrobial Action

Bombinins
- amphibians

Cecropins
- insects
- mammals

Dermaseptins
- amphibians

Magainins
- amphibians

Defensins
- plants
- insects
- mammals → HBD-2, -3

Cathelicidins
- mammals → LL-37

Granulysin

Human skin

Over 800+ peptides known

For review see Nature (2002) 415:389-395 and others

In Skin, Injury or Infection Triggers Antimicrobial Peptide Expression

Normal skin

Strep infection

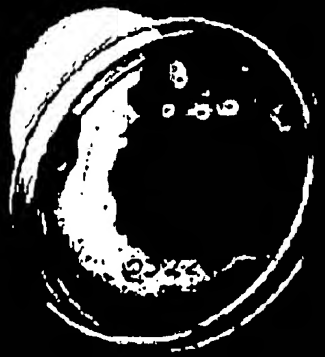
12 hr Wound

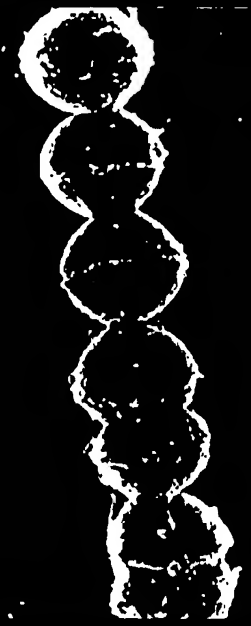
Brown staining= cathelicidin peptide immunoreactivity

J Invest Dermatol (2001) 117:91-97

Is an increase in antimicrobial peptides important?

Group A Streptococcus (*S. pyogenes*)





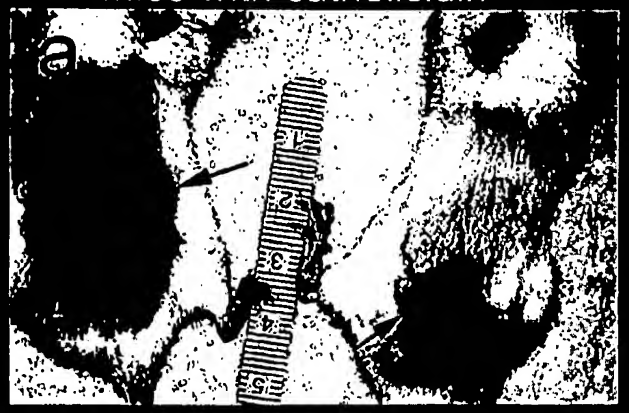
Sensitive to Cathelicidins

CRAMP: MIC = 2 - 8 μM

LL-37: MIC = 8 - 24 μM

In mice, cathelicidin knock-outs are more susceptible to Invasive Group A *Streptococcus*

Mice with cathelicidin

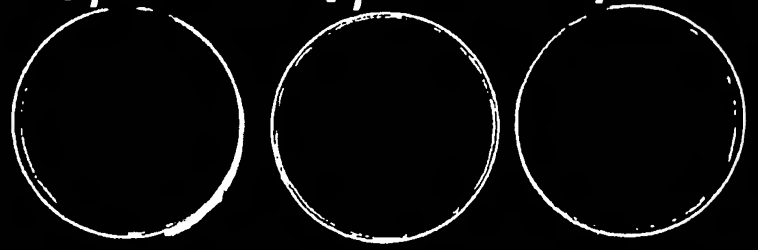


Mice with no cathelicidin

day 7

Lesion culture at 7 day

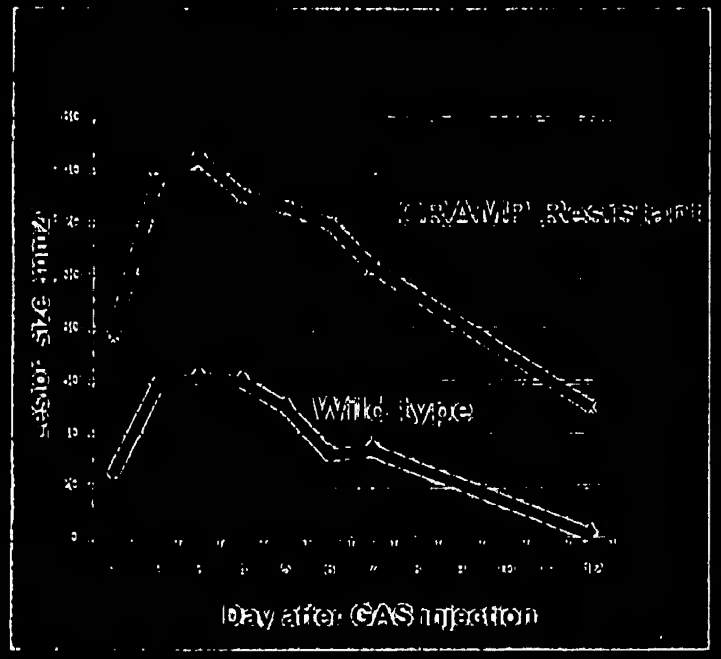
+/+ **+/-** **-/-**

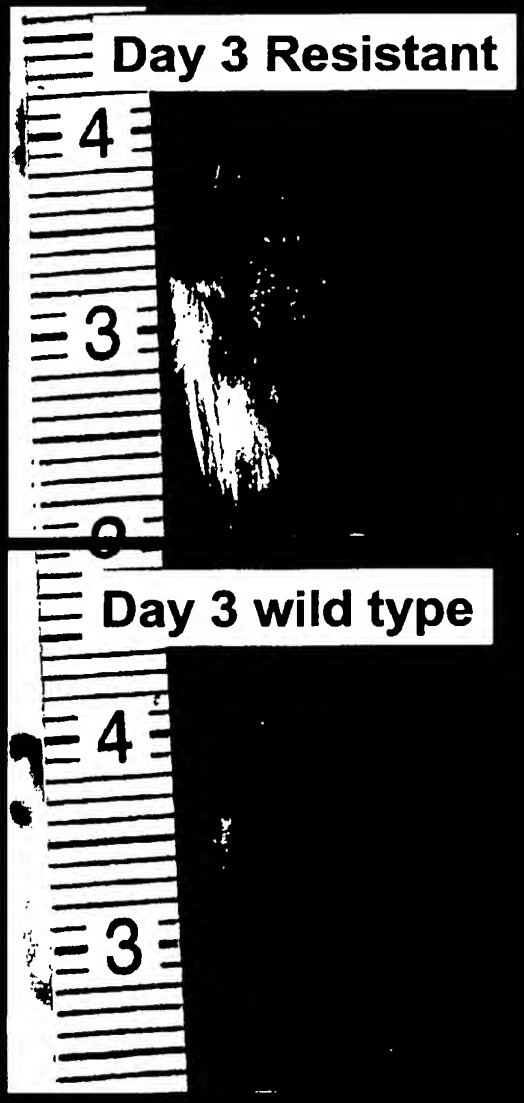


Nature, (2001) 414:454

Bacteria (GAS) that are cathelicidin resistant are more pathogenic

Lesion size

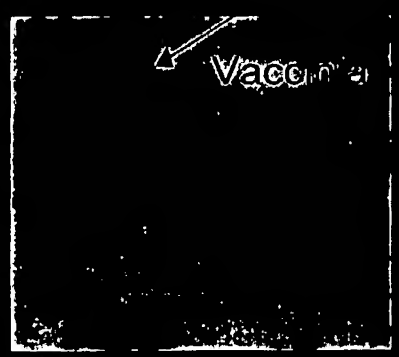




Nature, (2001) 414:454

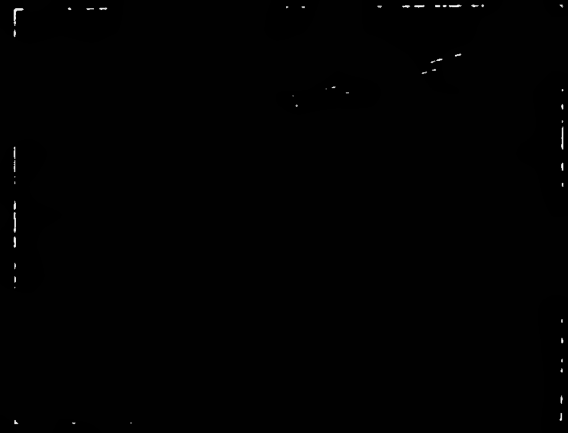
Cathelicidins also provide defense against viral infection

No LL-37

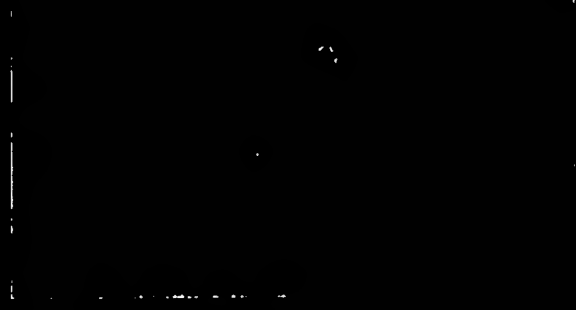


LL-37 5 μM

LL-37 (μM)	Altered virions/ virion number (%)	
0	1/23	(4%)
5	19/28	(67%)
25	27/30	(90%)



No Lesion 15/16 Control Mice



Vesicular Lesion 4/6 CRAMP --/--

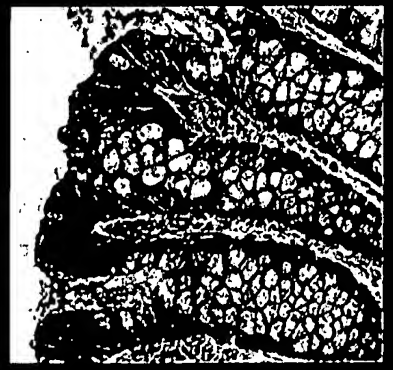
Conclusion:

The presence of antimicrobial peptides is essential
for normal defense against certain bacterial and viral skin infections

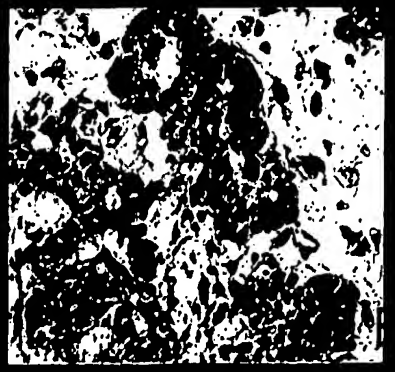
How are the cathelicidin antimicrobial peptides used by the skin?

Cathelicidins are expressed at the interface

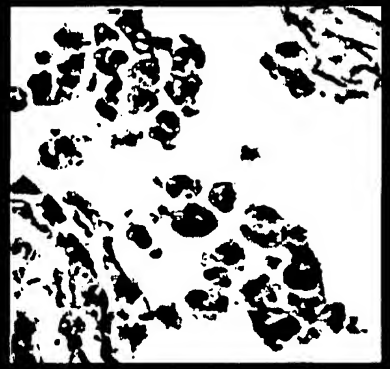
Skin



Colon



Salivary Gland



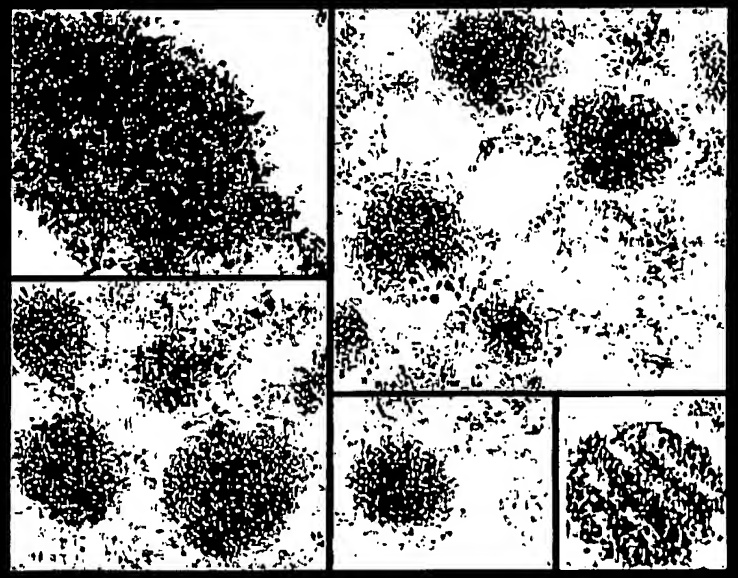
Neutrophil

Mast Cell

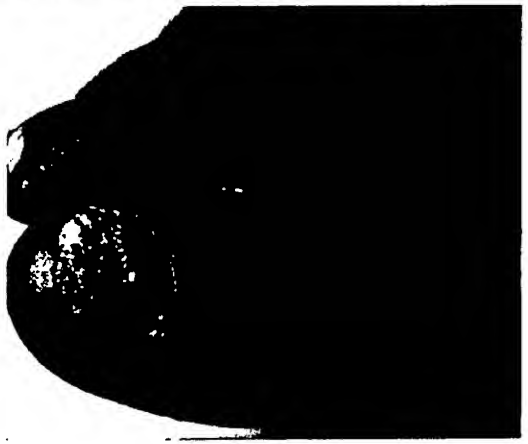
Caths are stored in Keratinoctye granules

FITC Anti-LL37

Anti-LL37 immuno gold EM

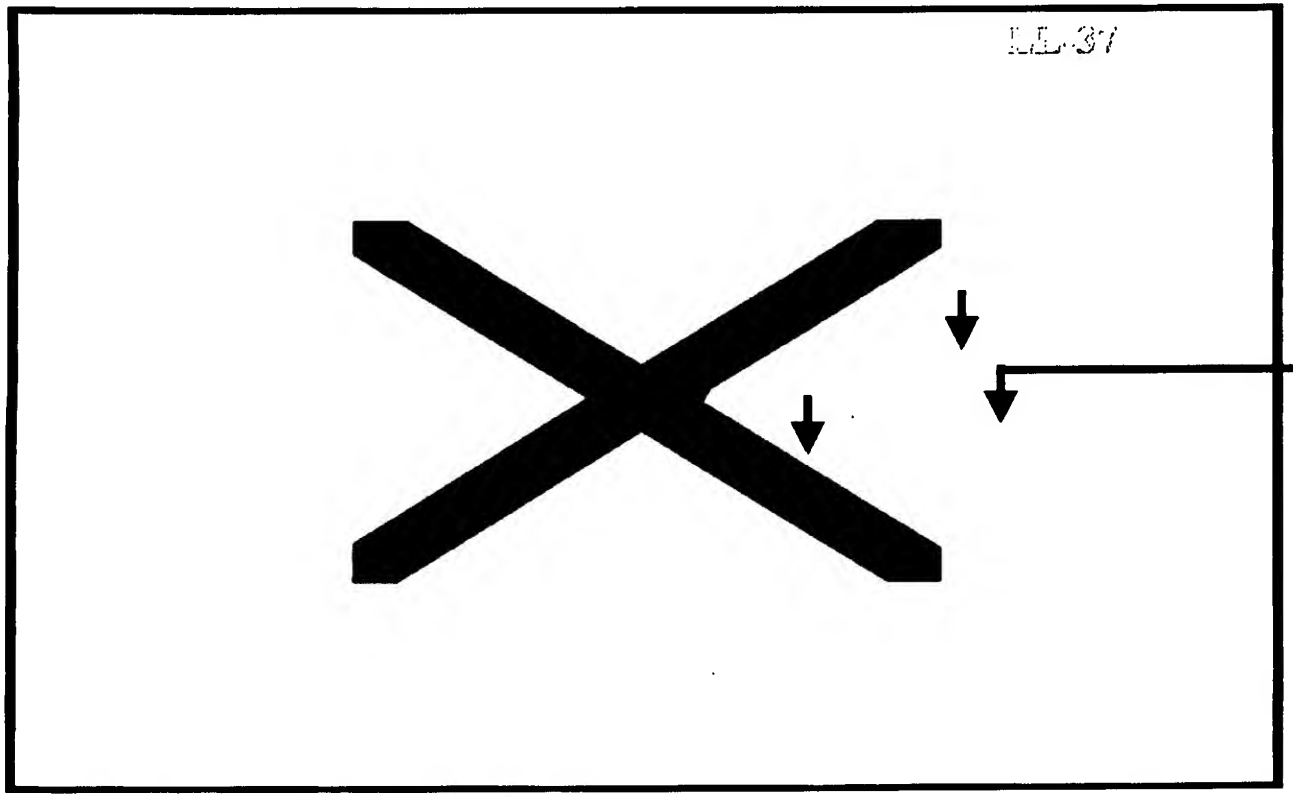


Caths are expressed in the nail apparatus



Is this an answer to normal nail defense?

Cathelicidin peptides discovered in sweat with increased activity



Eccrine gland and duct stain for cathelicidin

New bioactive forms

Kill bacteria better after processed by enzyme in sweat

Work of Dr. M. Murakami, now a Dermatology resident at Asahikawa

Does this correlate with human disease?

Atopic patients are susceptible to infection and have inflammation.
What is their expression of antimicrobial peptides?

Eczema Herpeticum

Eczema Vaccinatum

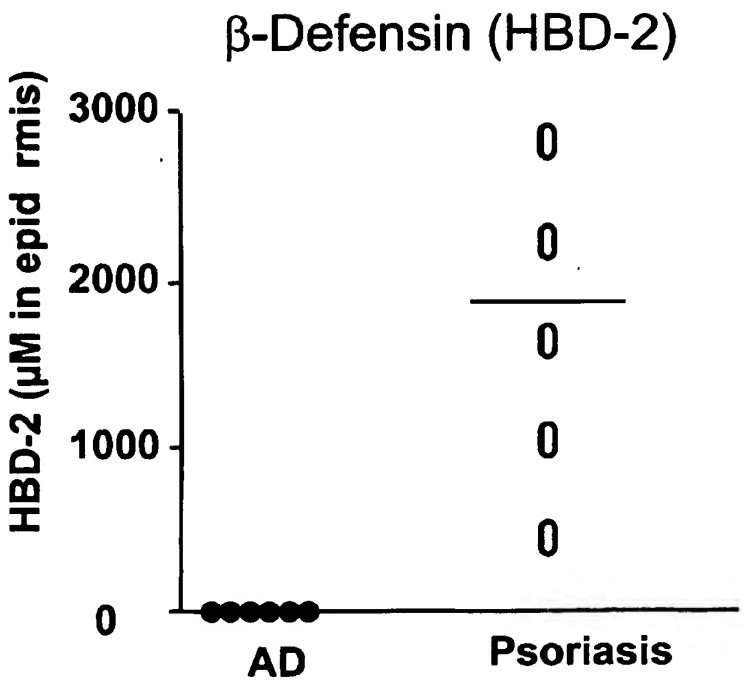
Impetigo

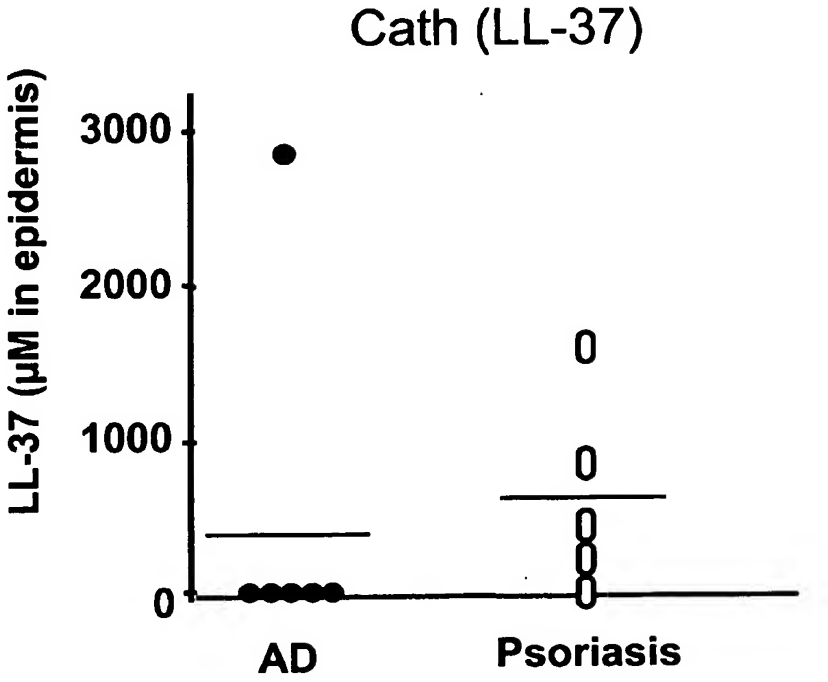
HSV, VZV

Staph
Strep

Cow pox

Compared to psoriasis, atopic patients do not respond to inflammation with a increase in antimicrobial peptides





Ong et al. (2002) N Engl J Med 347:1151-60

Work of Dr. T. Ohtake, now faculty in Medicine at Asahikawa

Conclusions

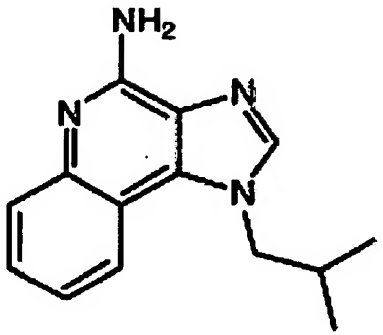
Cathelicidins are produced by several cells in the skin.
A lack of normal production correlates with disease

Psoriasis
Overproduction ➡ Protection

Atopic Dermatitis
Underproduction ➡ Infection

Can our increased understanding of innate immunity and antimicrobial peptides be used for therapy?

Imiquimod:



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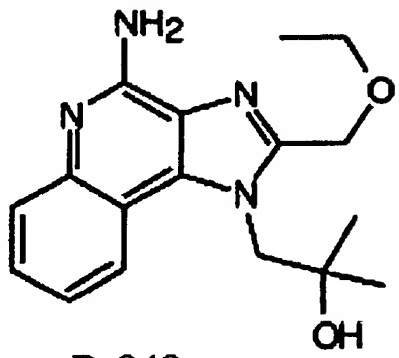

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PR-39 expression in wound lesion

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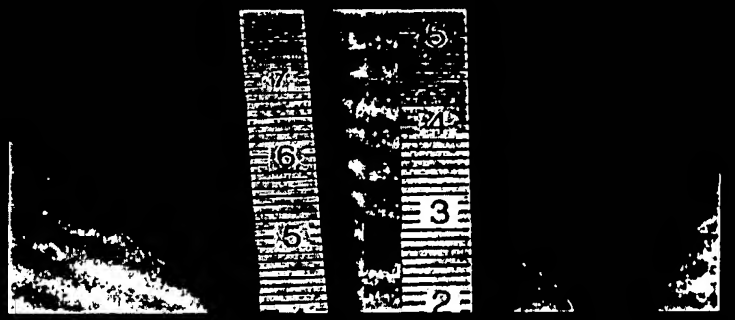

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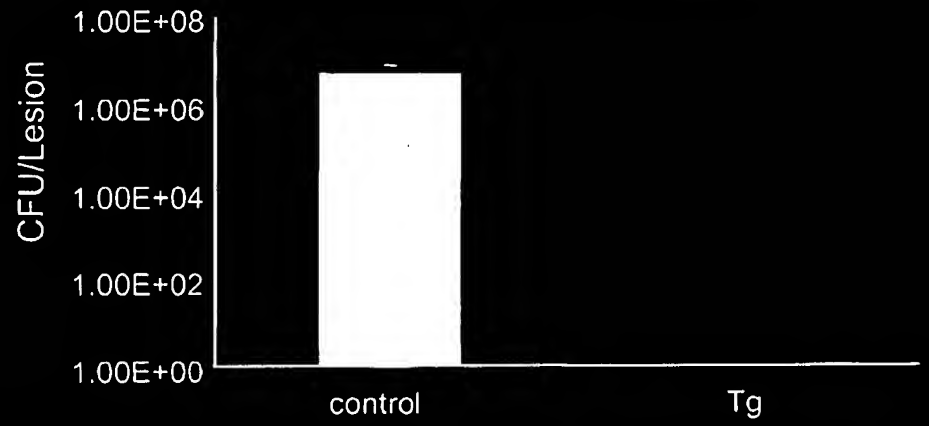
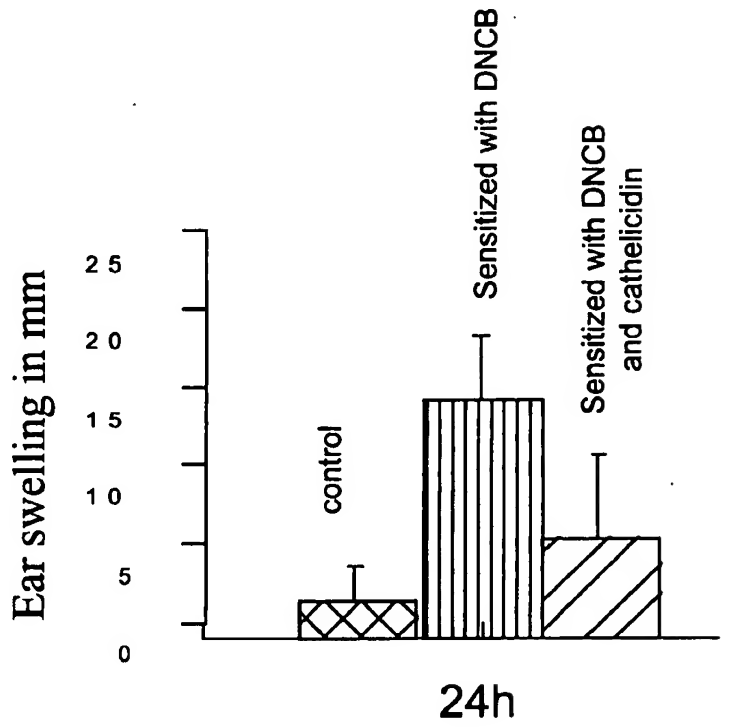


Human trials are underway

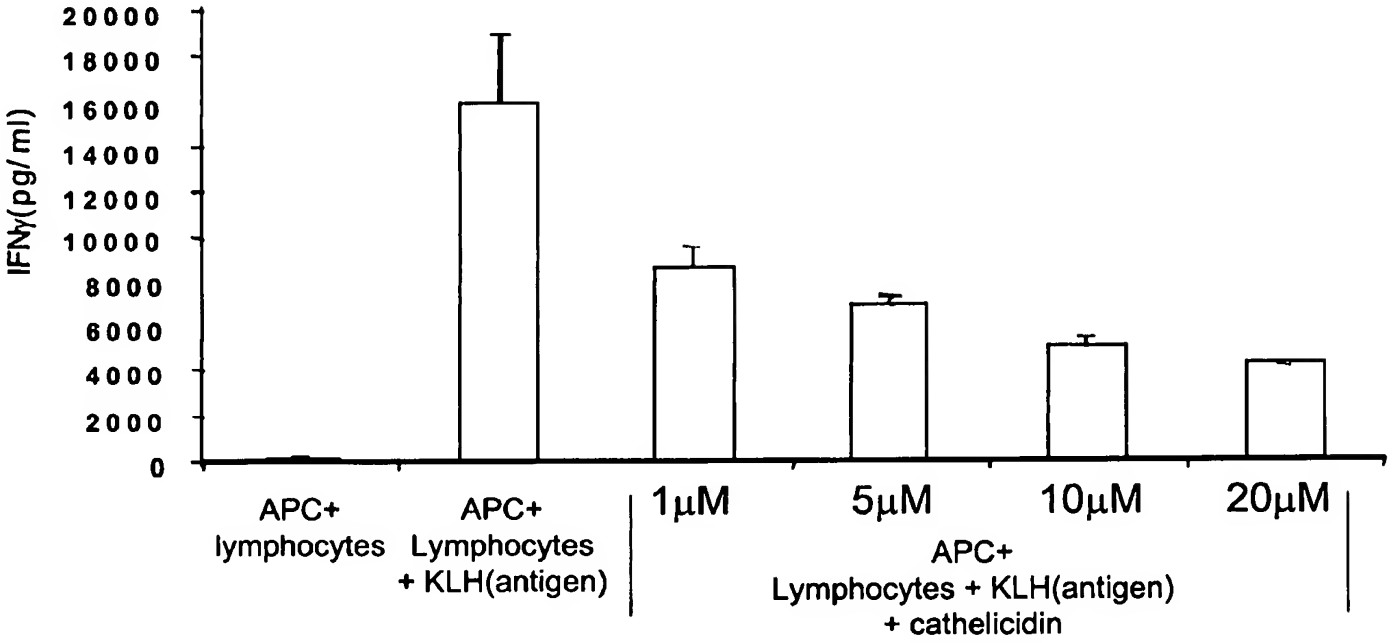
Route	Peptide	Company	Application	Stage
Topical	MSI-78	Maganin	DM ulcers	Phase 3
Topical	MBI-226	Micrologix	Cath infections	Phase 3
Topical	MBI-594		Acne	Phase 2
Oral	Protegrin	Intrabiotics	Mucositis	Phase 3
Oral	Histatin	Demegen	Gingivitis	Phase 2
Systemic	Heliomycin	Entomed	Antifungal	Pre-clinical
Systemic	Lactoferricin	AM Pharma	Antibacterial	Pre-clinical
Systemic	BPI	Xoma	Meningitis	Phase 3

However,
Antimicrobial Peptides do more than kill microbes!

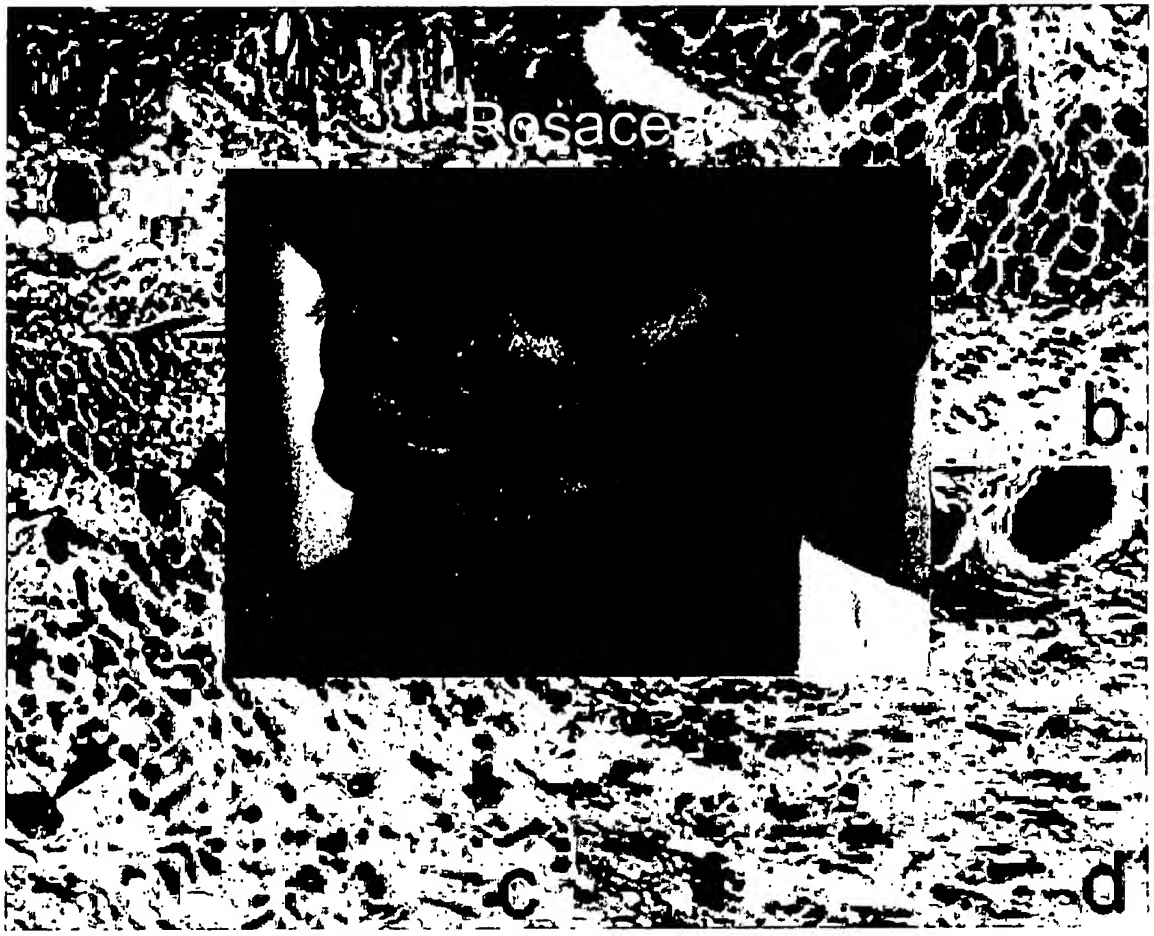
Cathelicidin blocks allergic contact dermatitis in mice



Cathelicidin blocks antigen presentation



But chronic administration of cathelicidin is proinflammatory



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Antimicrobial Peptides in the Skin

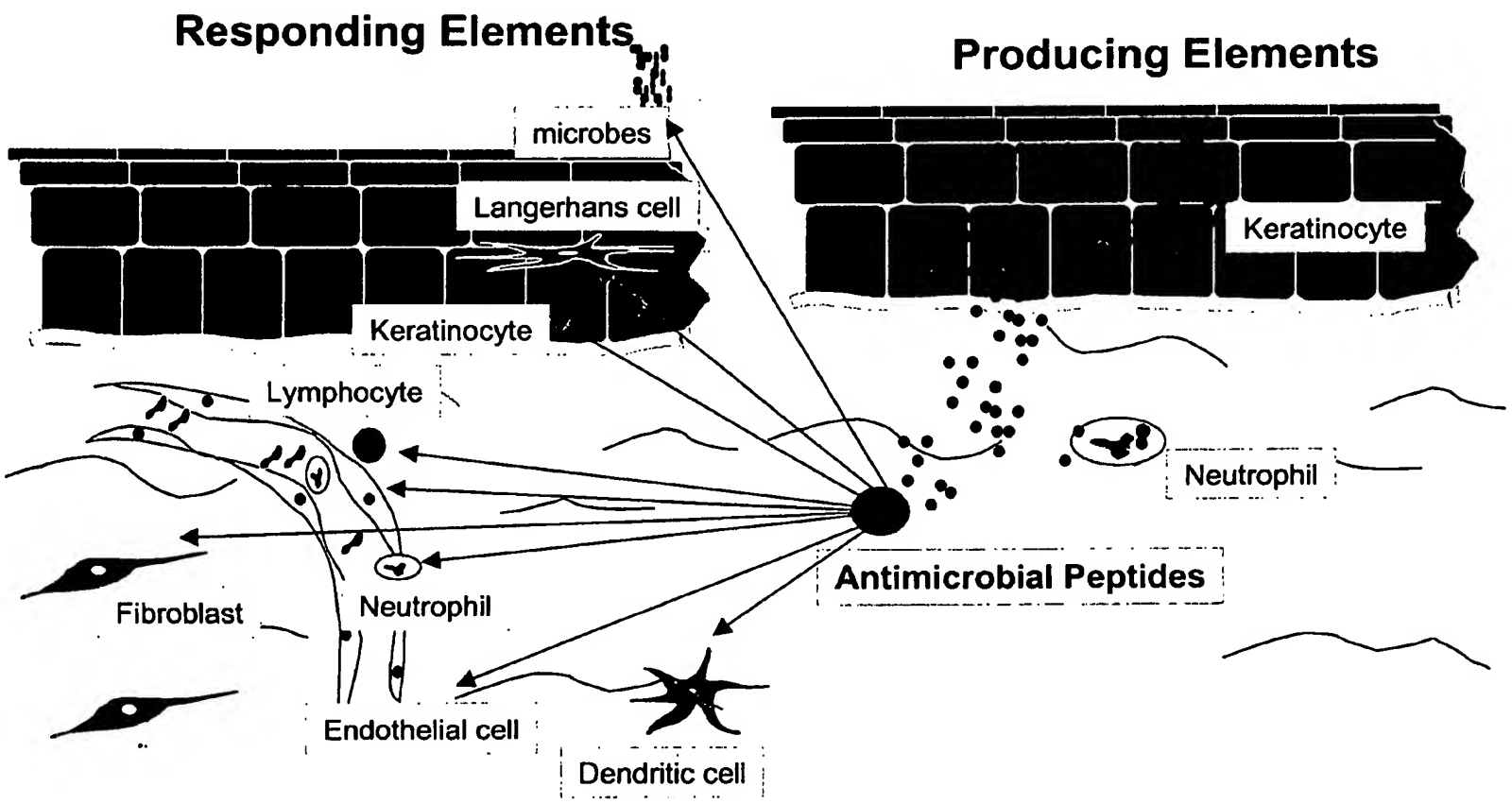

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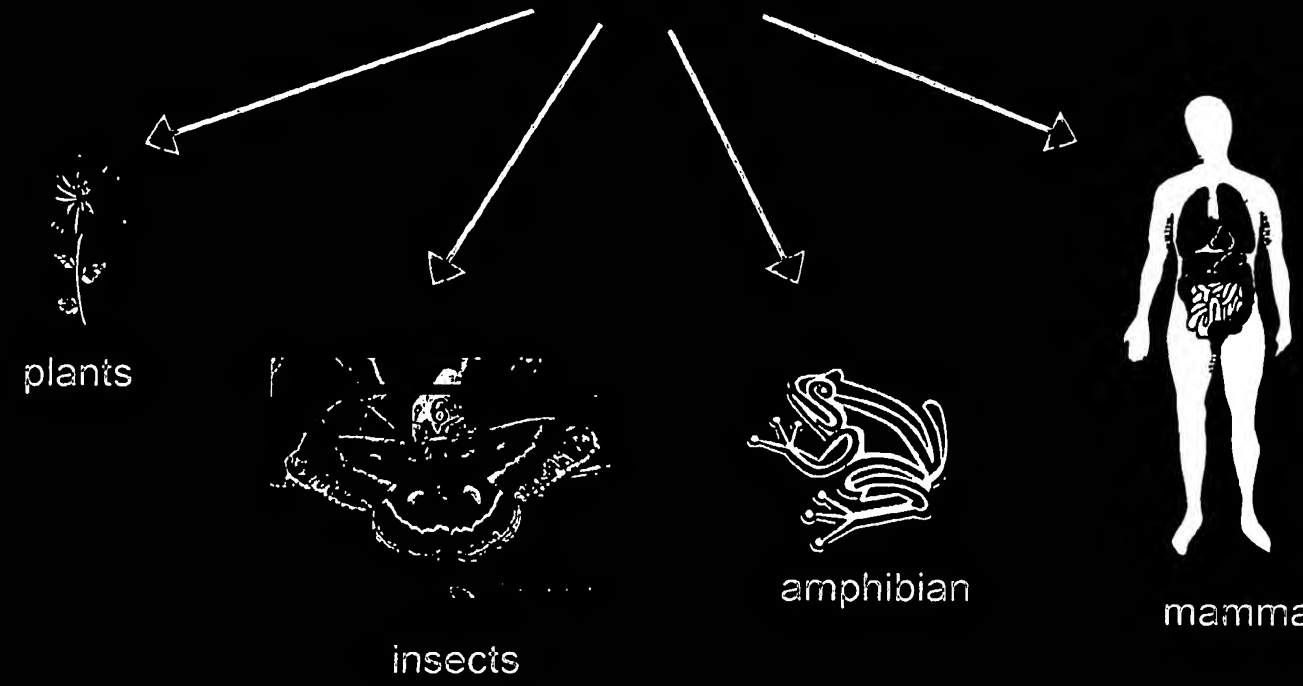
FIGURE 19 - 37pg

Antimicrobial Peptides and Innate Defense of the Skin

Richard L. Gallo, M.D., Ph.D.
Medicine and Pediatrics
U. of California, San Diego

Peptides with antibiotic activity are found throughout nature.



Over **800** peptides now known
Searches based on ability to kill microbes in culture

Multiple antimicrobial peptide gene families (partial list)

- Bombinins
  - amphibians
- Cecropins
  - insects
  - mammals
- Dermaseptins
  - amphibians
- Magainins
  - amphibians
- Defensins
  - plants
  - insects
  - mammals
- Cathelicidins
  - mammals
  - fish

www.bbcm.univ.trieste.it/~tossi/pag1.htm

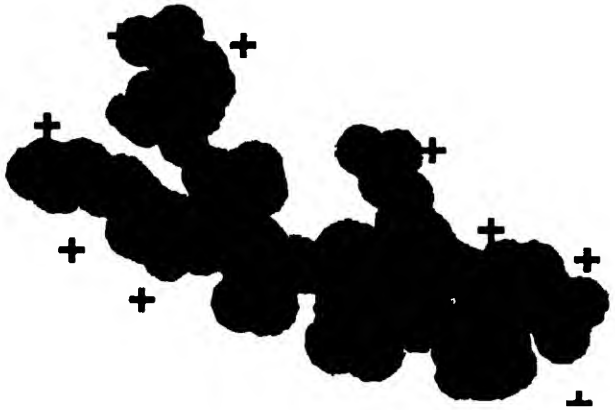
Antimicrobial peptides are often cationic and amphipathic

Human alpha defensin

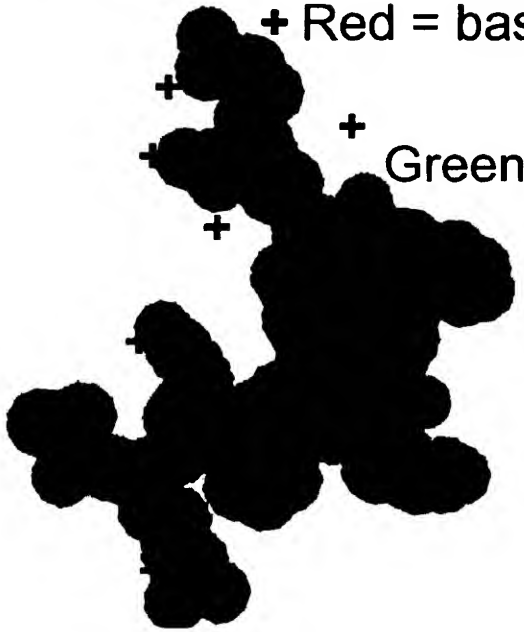
Frog Maganin

+ Red = basic amino acids

Green=hydrophobic

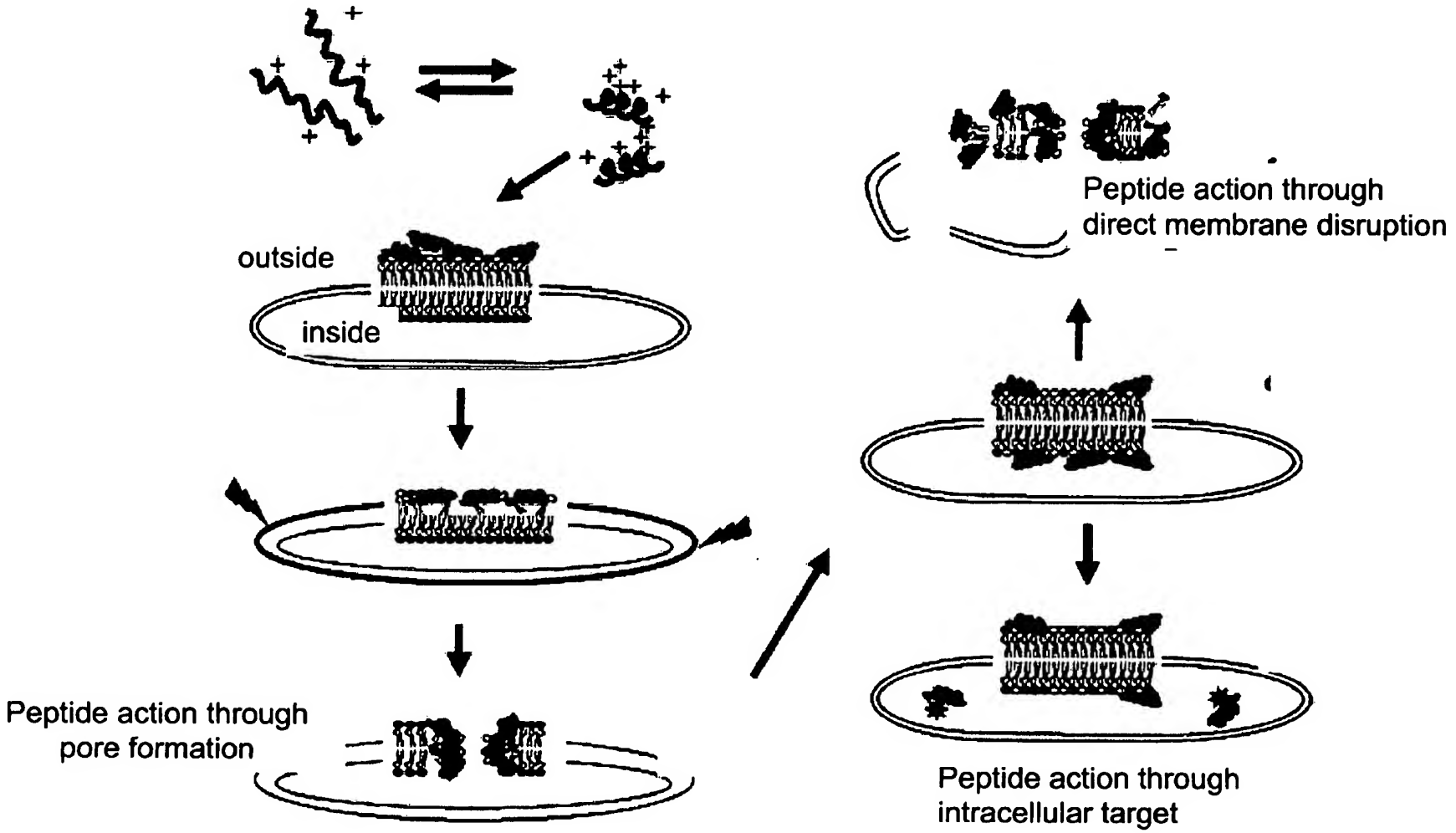


Pig Cathelicidin (Protegrin)

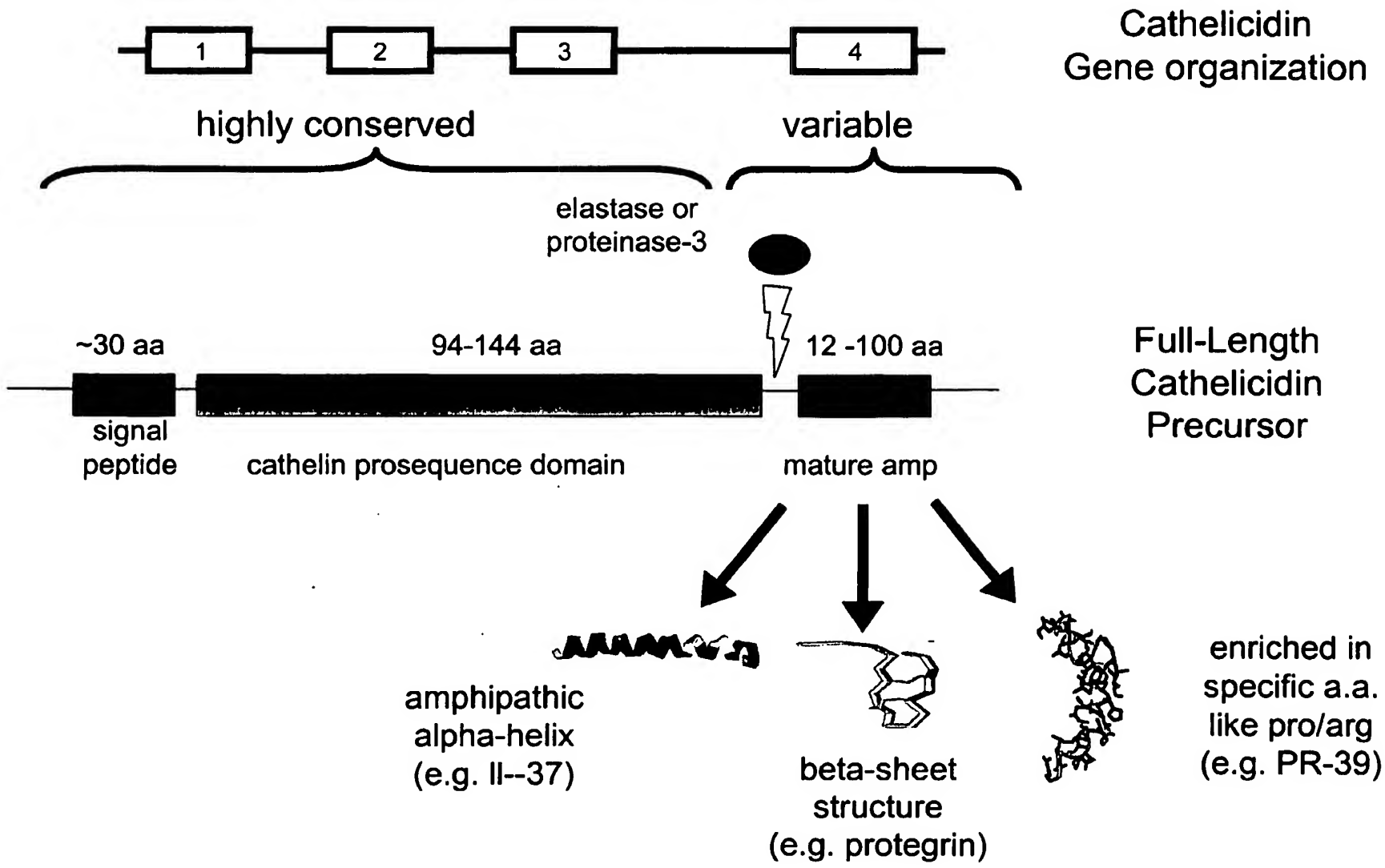


Pig Cathelicidin (Indolicidin)

Antimicrobial action is dependent on membrane activity

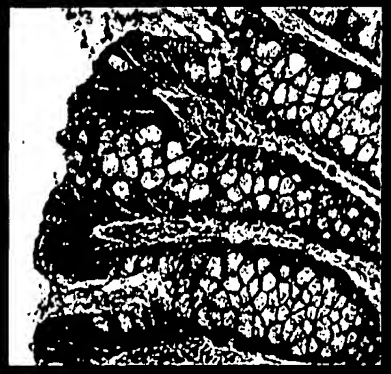


Cathelicidins:

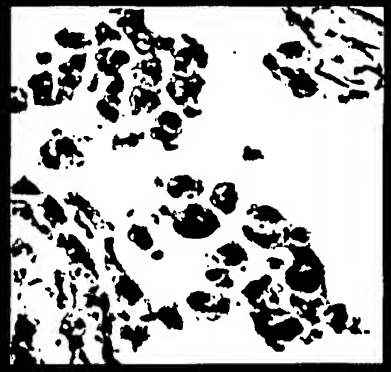


Caths are expressed at the interface

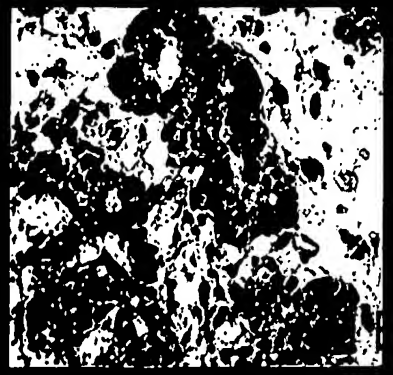
Skin



Colon



Neutrophil



Salivary Gland

Mast Cell

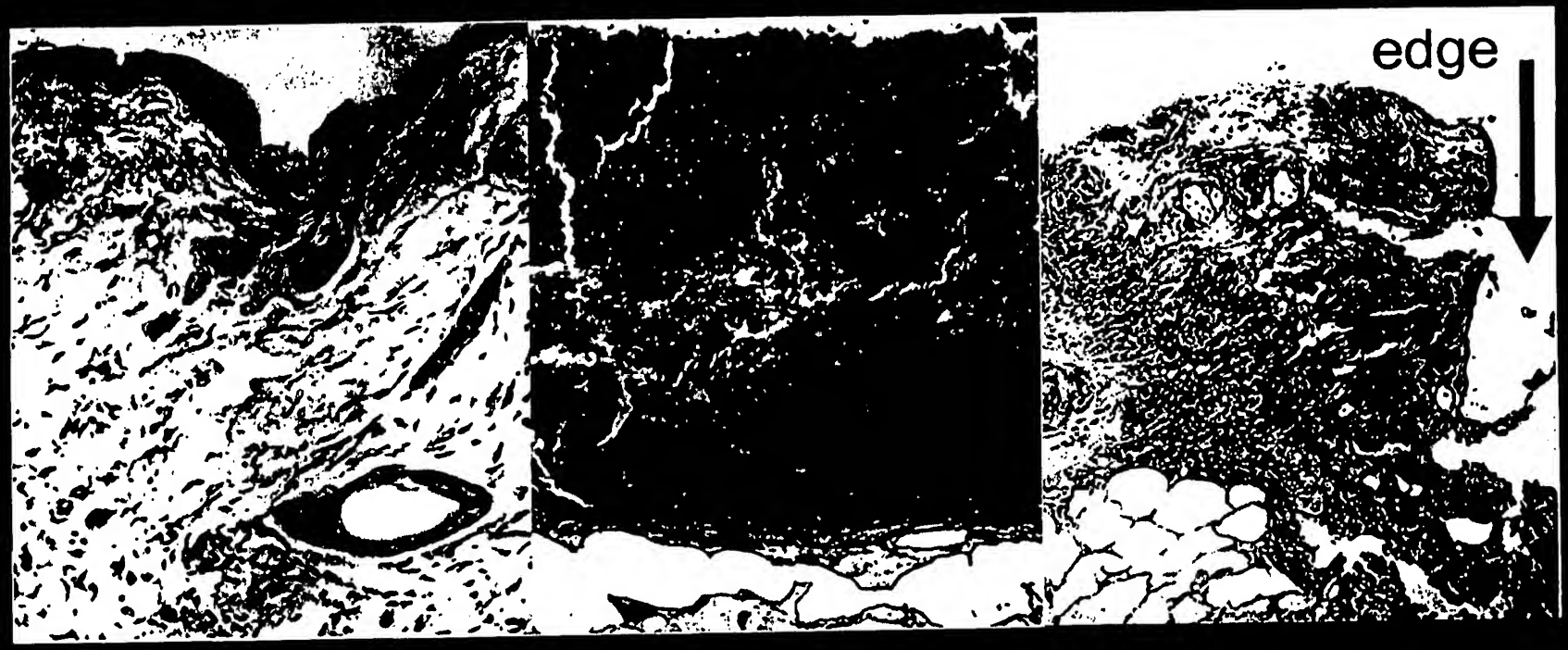
Sweat Gland

In Skin, Injury or Infection Triggers Cath Expression

Normal skin

Strep infection

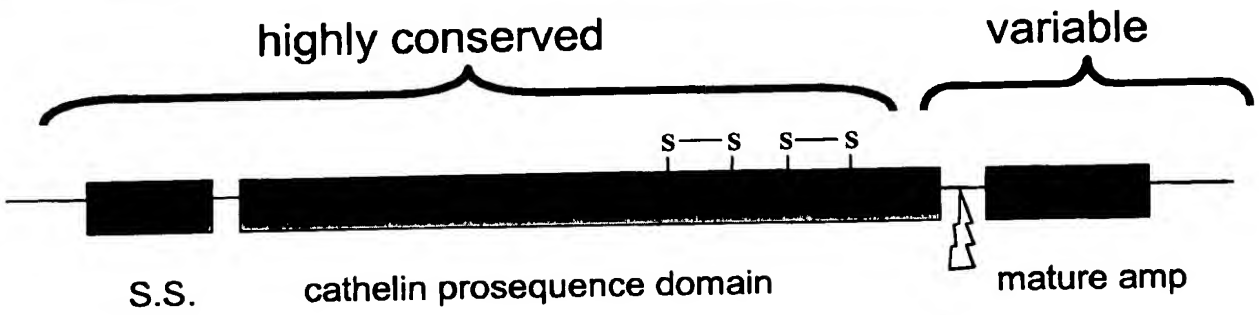
12 hr Wound



Brown staining= cathelicidin peptide immunoreactivity

J Invest Dermatol (2001) 117:91-97

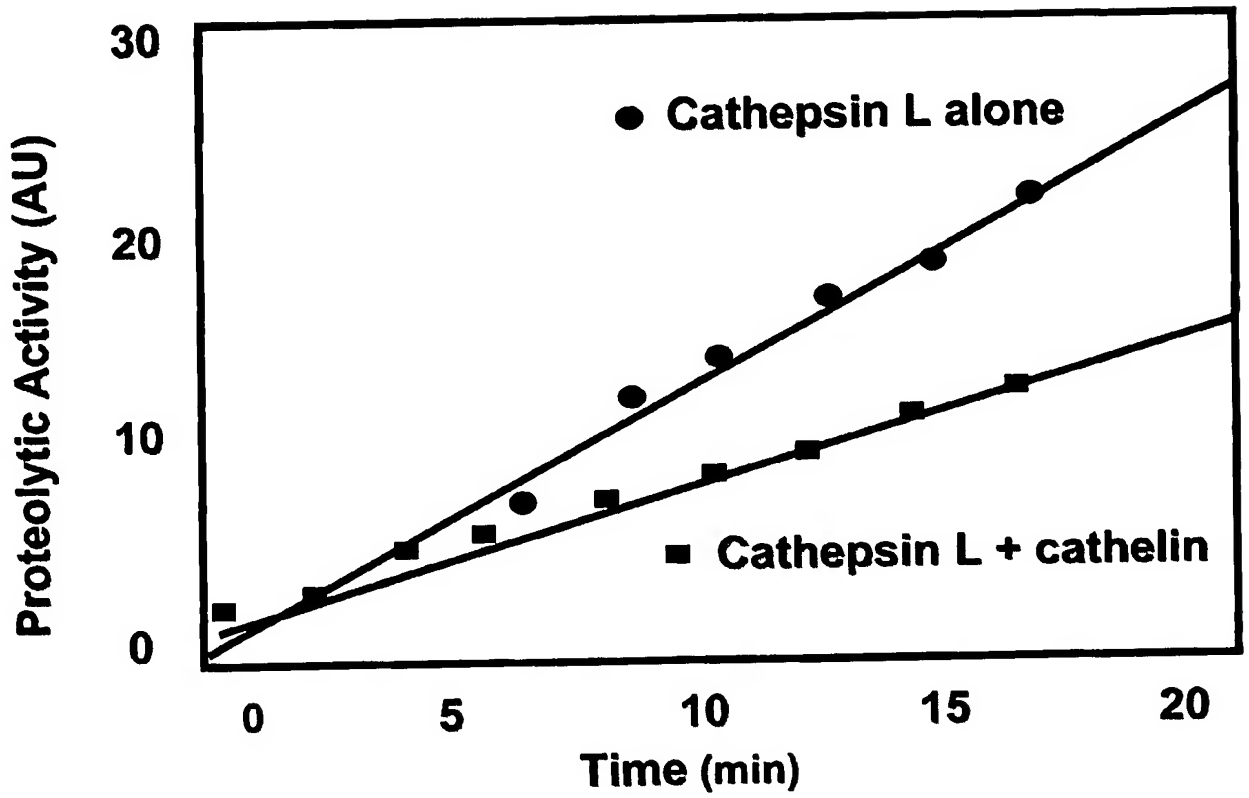
Cathelicidins: Prodomain Function?



Human cathelin Domain

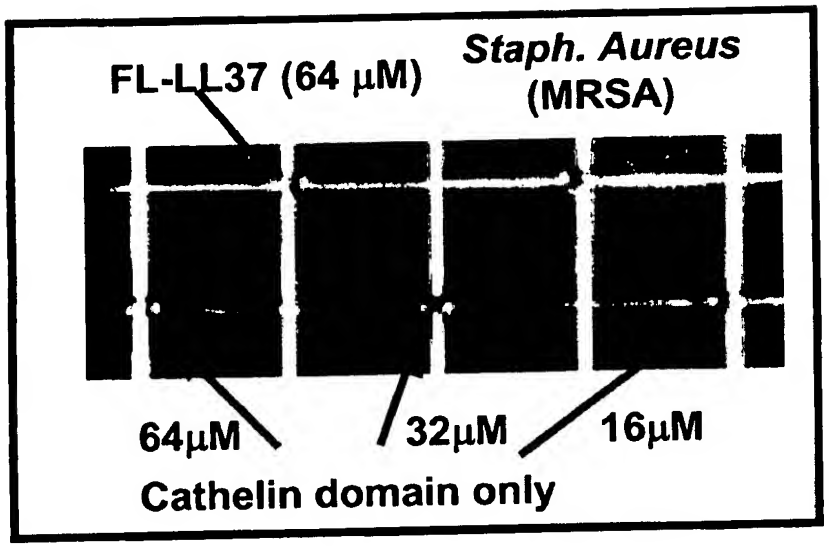
Recombinant expression in *E. coli*

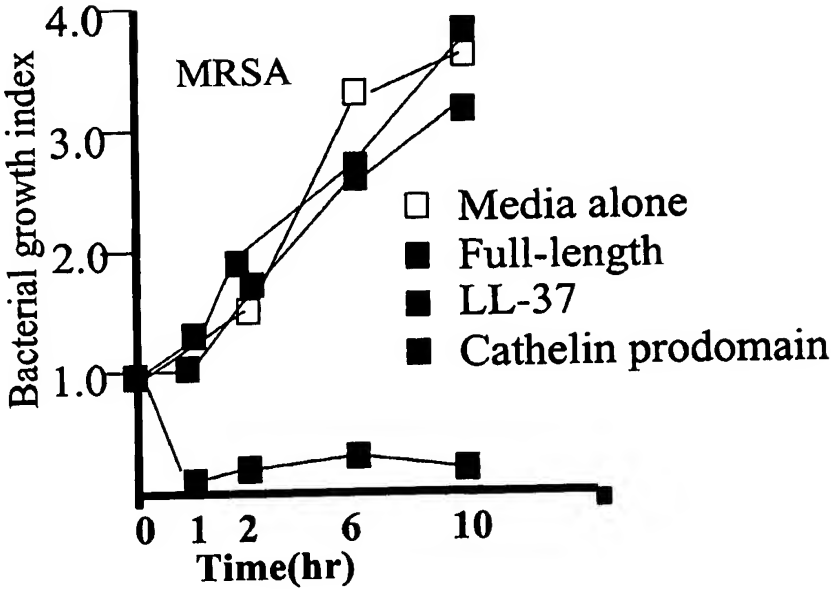
Cathelicidins: Prodomain Function



Human cathelin domain has Cysteine Protease Inhibitor activity

Cathelicidins: Prodomain Function

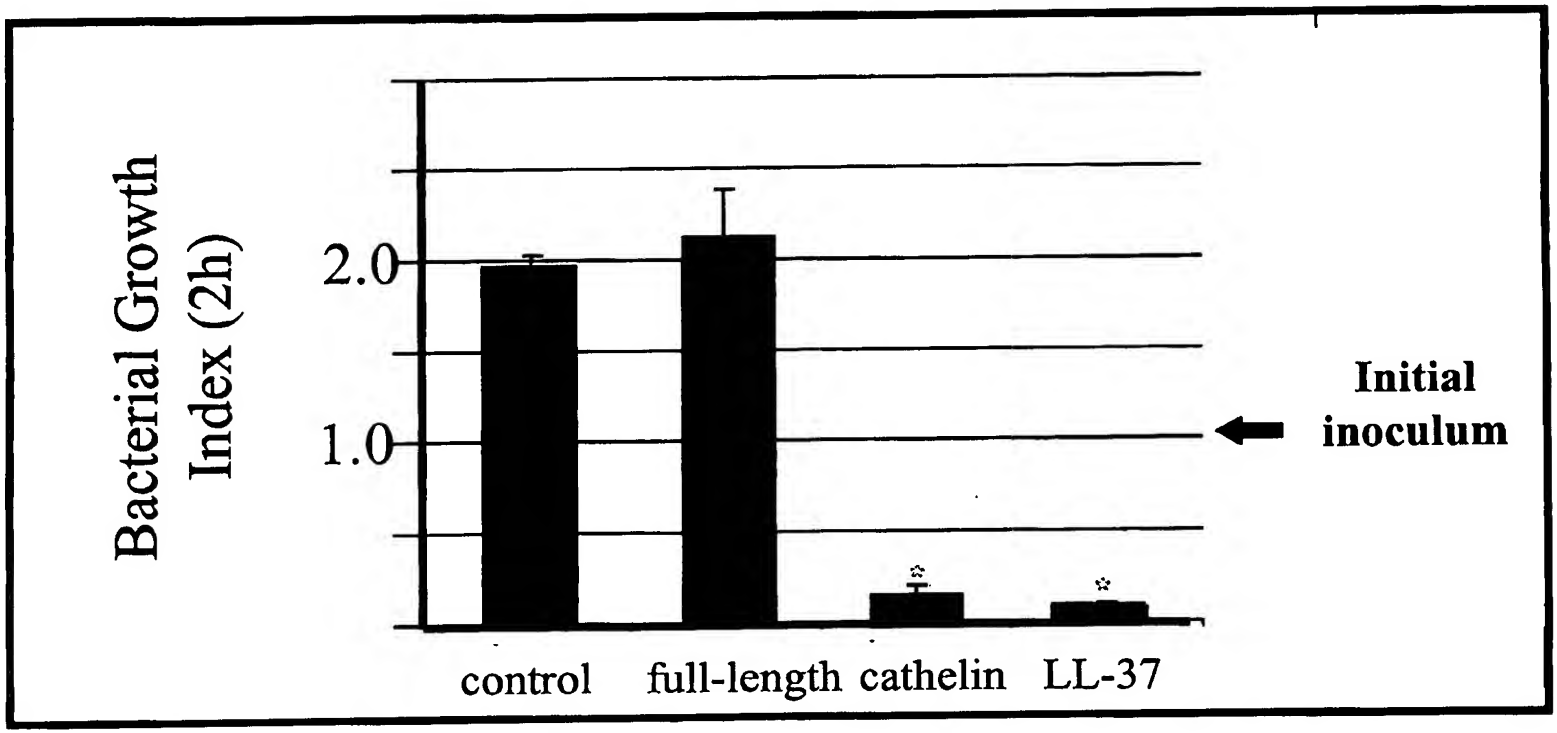




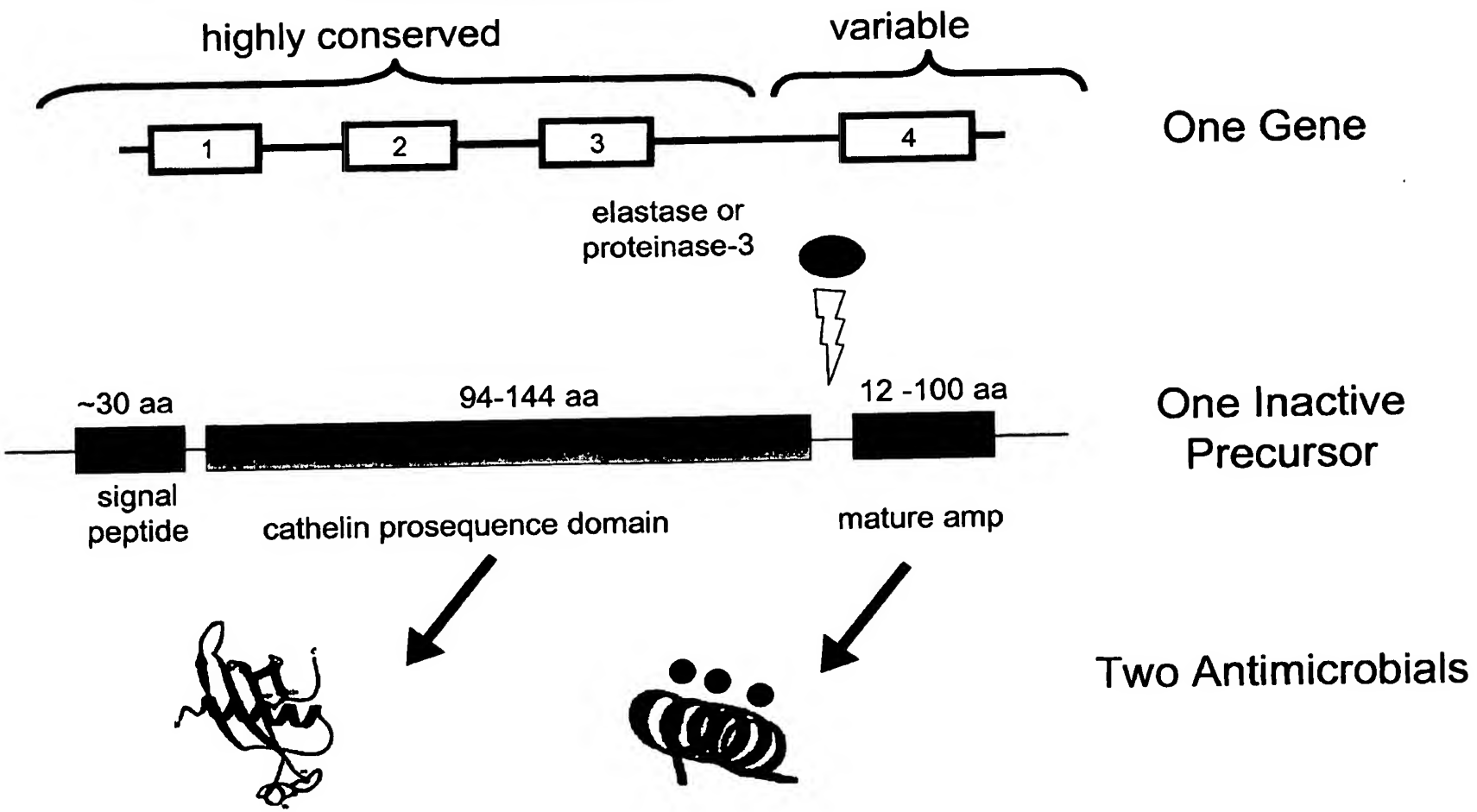
Human cathelin domain has independent antimicrobial activity

Keratinocytes with expressed cathelicidin gain function

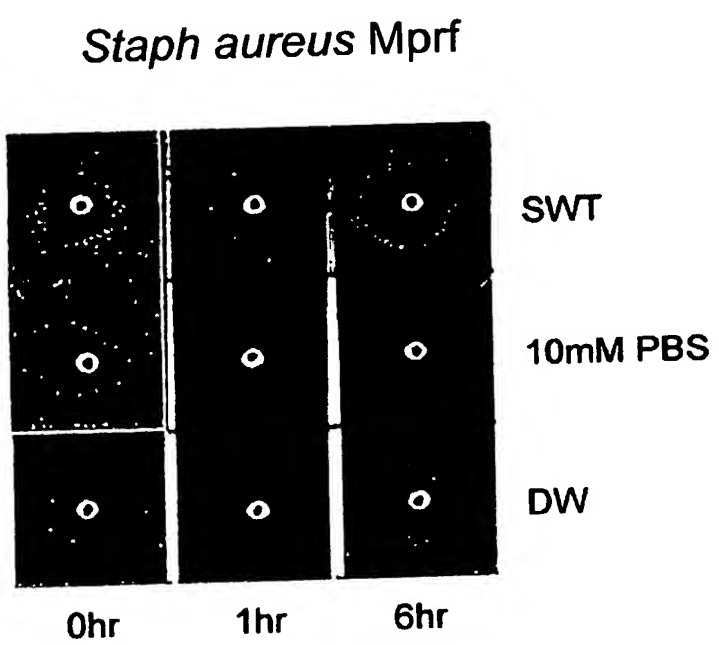
HaCat human keratinocyte cell line
Lentiviral expression system
Full-length, cathelin prodomain and LL-37 C-terminal AMP
Test bacterium: Cathelicidin-sensitive *S. aureus* mprF mutant

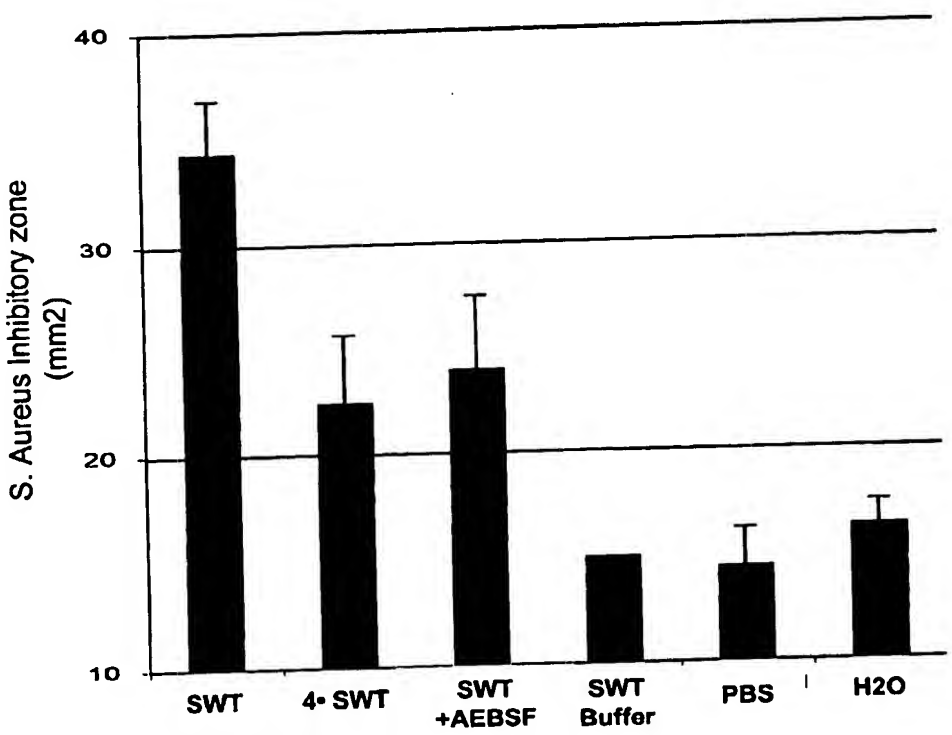


Cathelicidin: "Double Trouble" for Bacteria

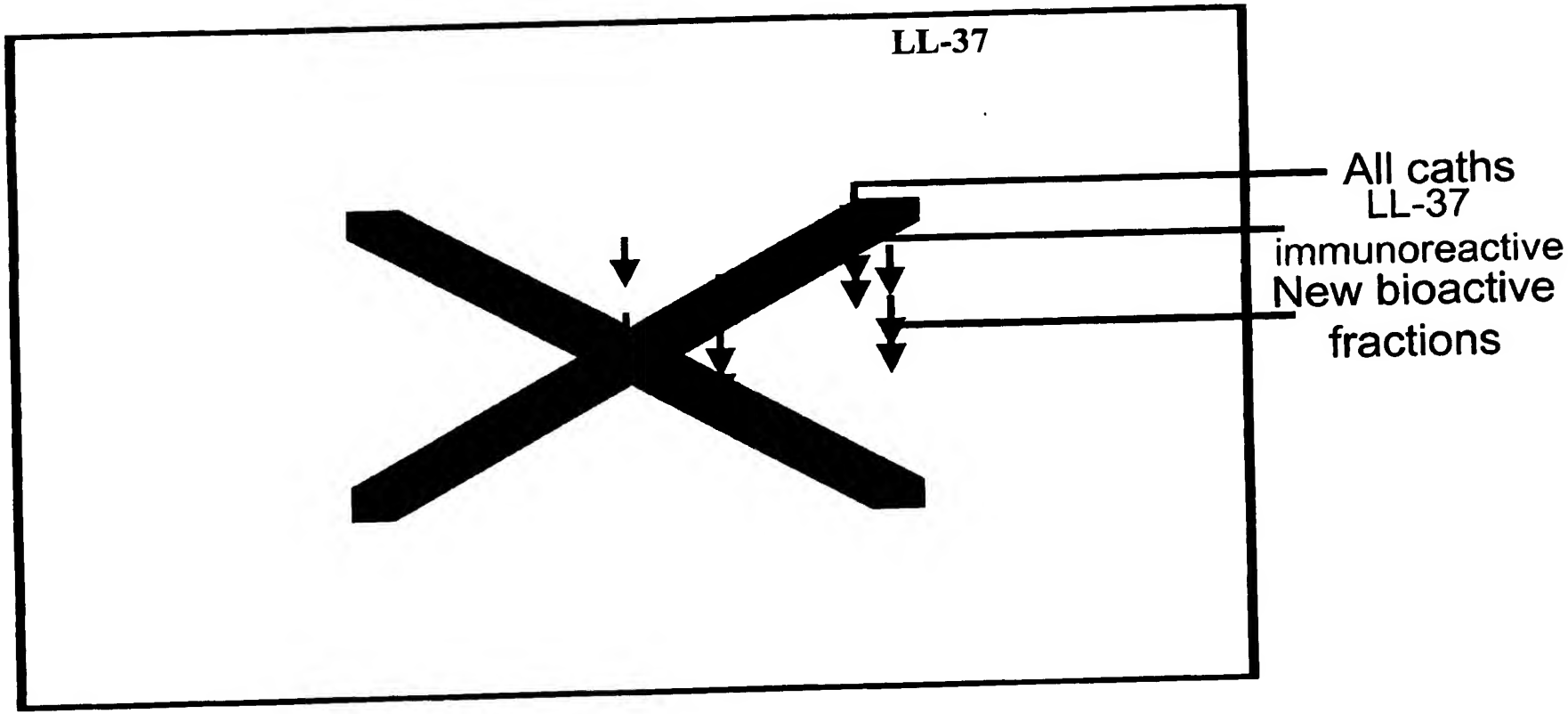


Human cathelicidin LL-37 increases activity at the skin surface

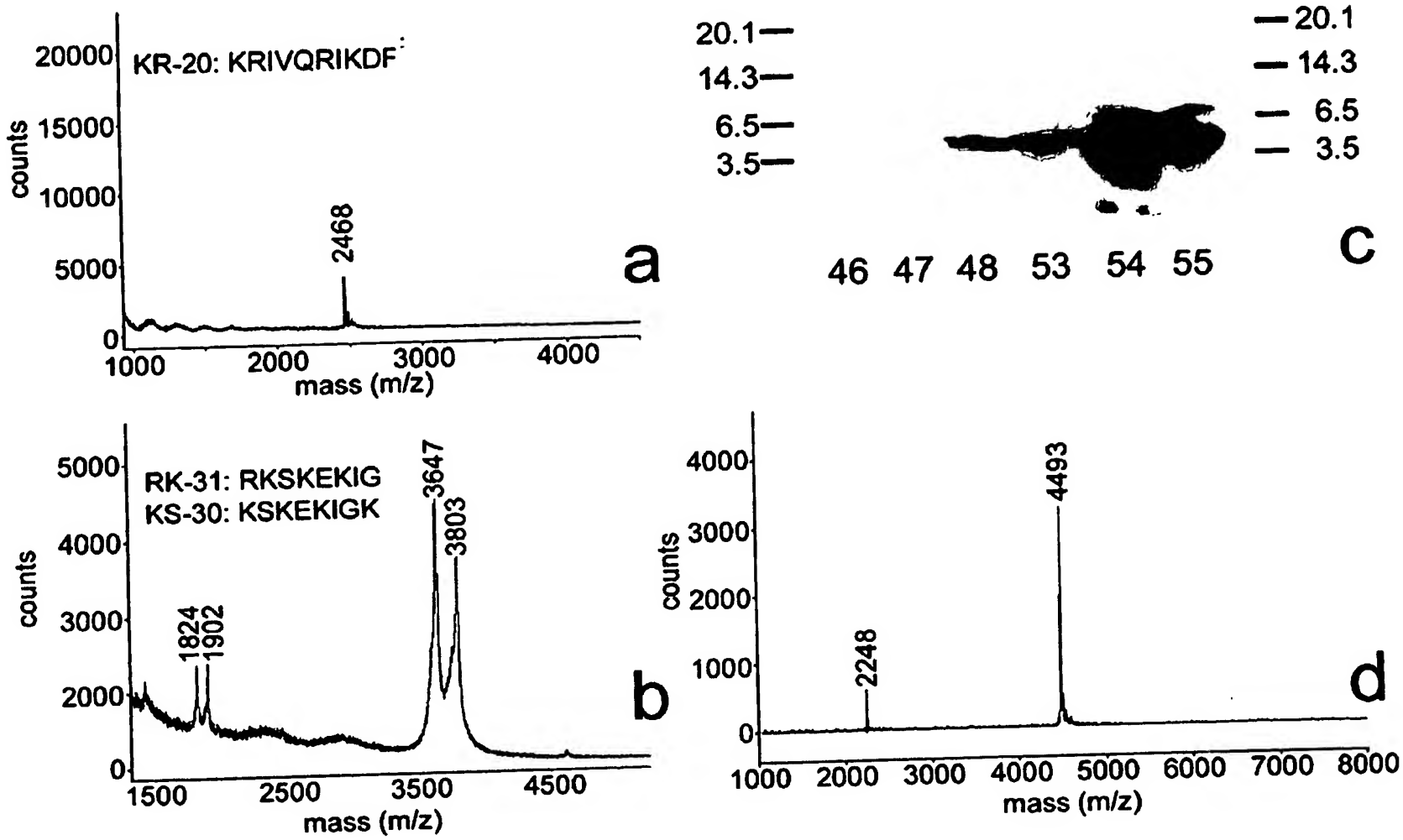




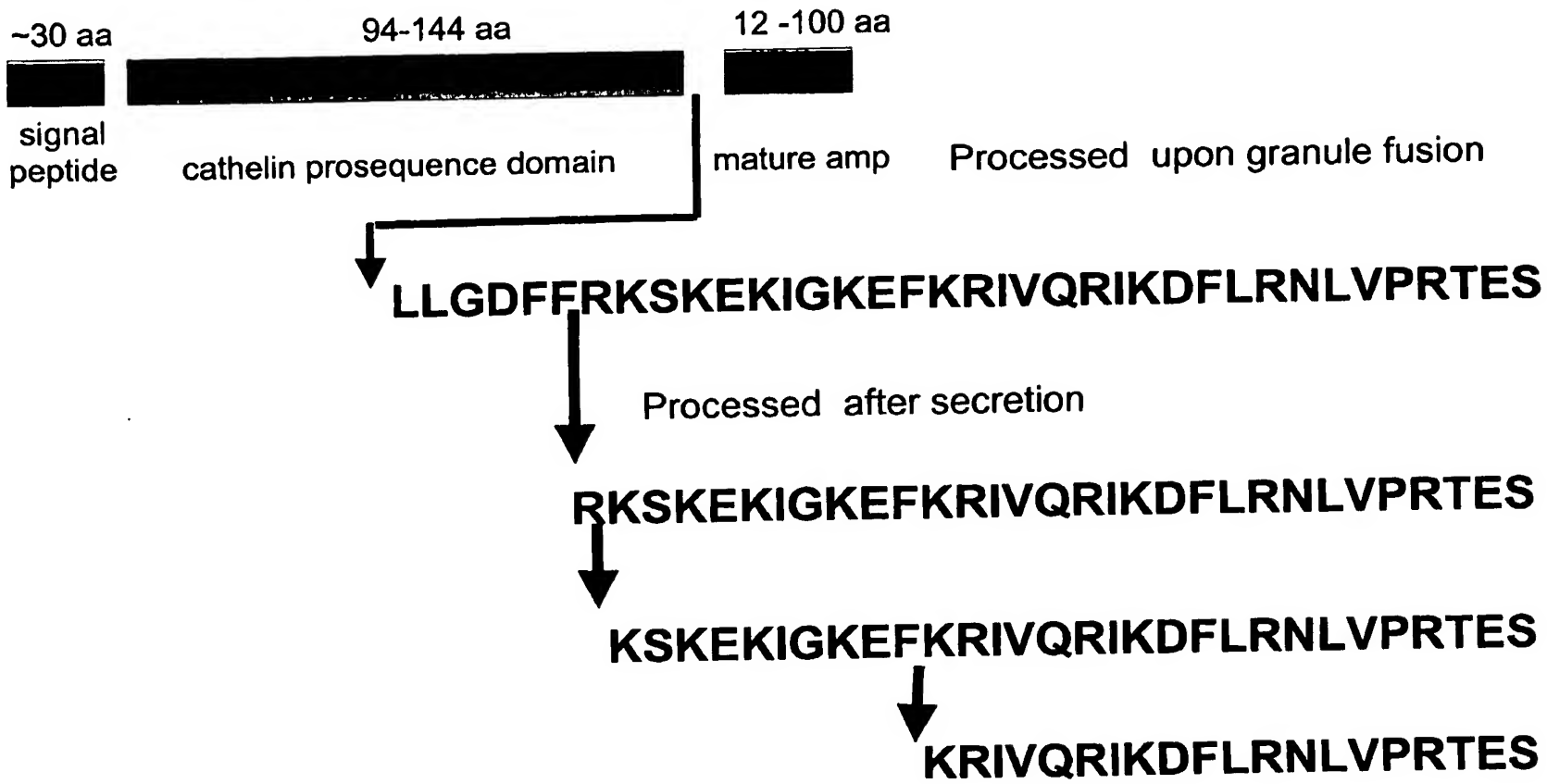
Detection of Cathelicidin peptides on the skin surface



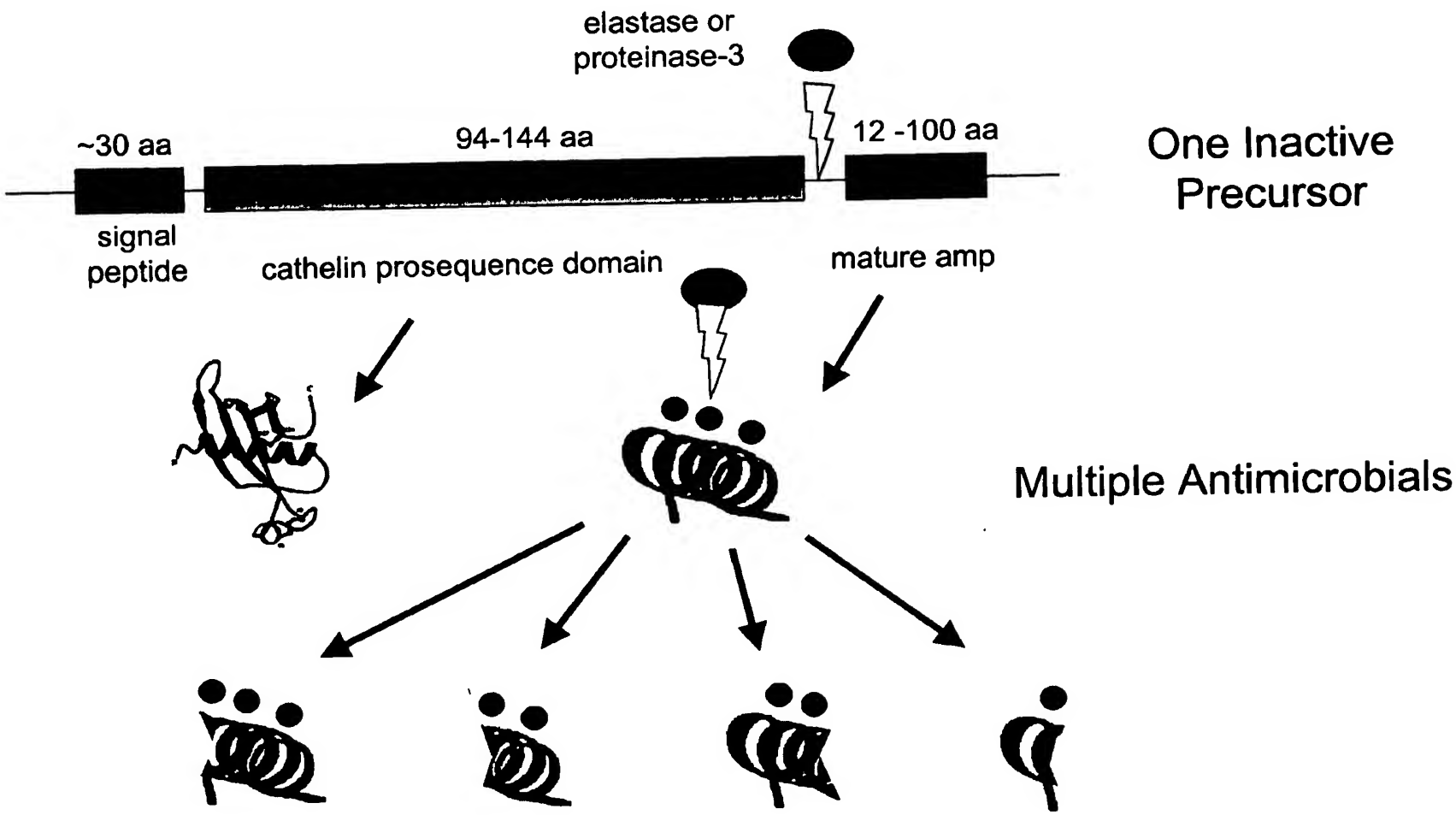
Identification of cathelicidin peptides on the normal skin surface



Processing of cathelicidin peptides on the normal skin surface

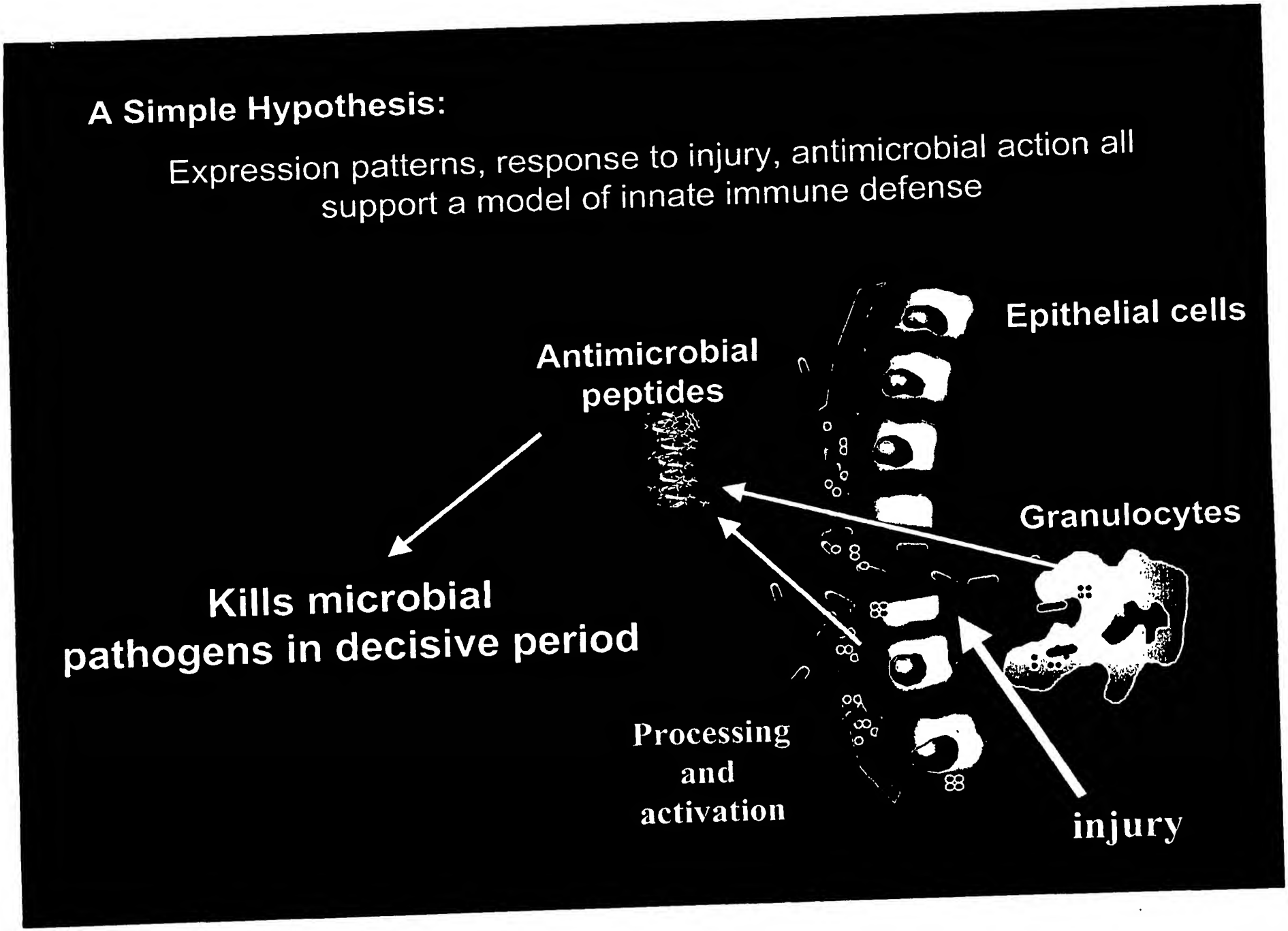


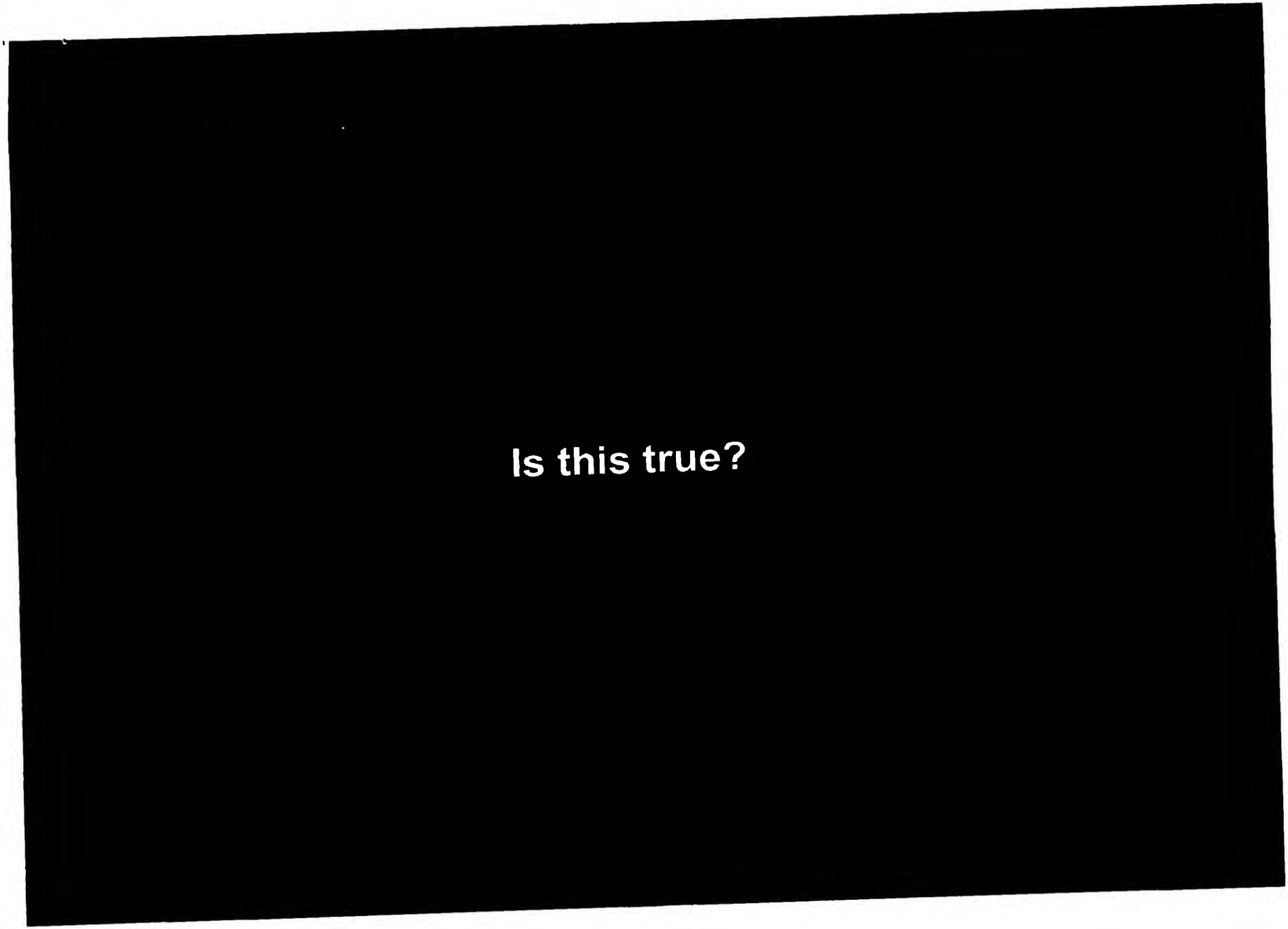
Cathelicidin: Single Gene , Many Antibiotics



A Simple Hypothesis:

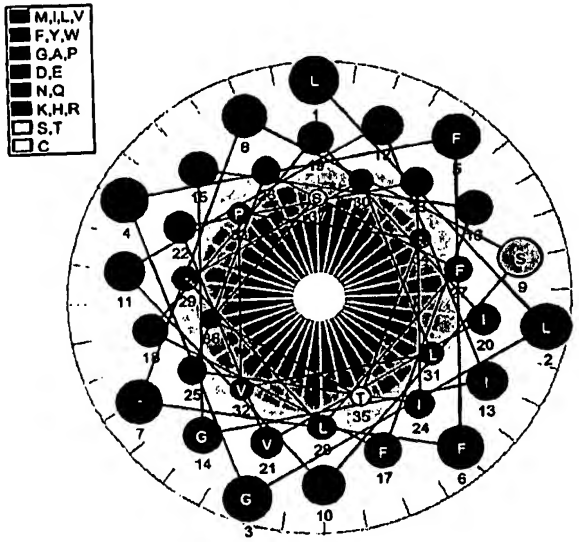
Expression patterns, response to injury, antimicrobial action all support a model of innate immune defense



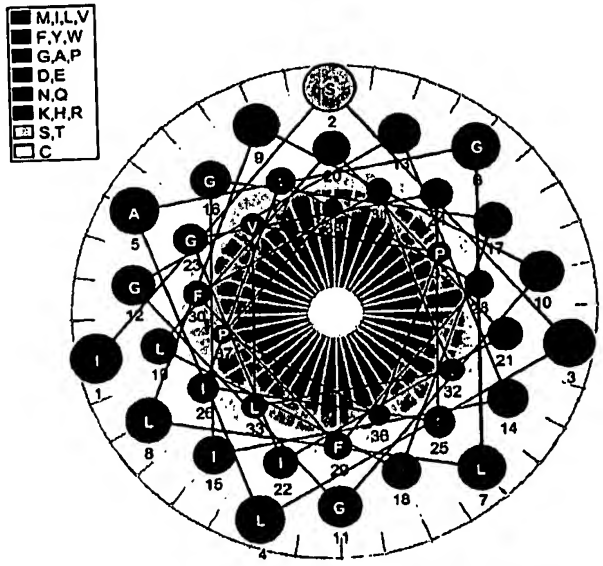

Is this true?

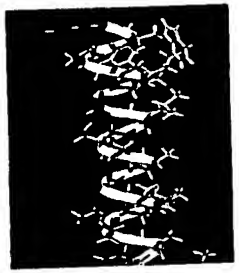
Cathelicidin Peptides: Man vs. Mouse

LL-37 (Human)

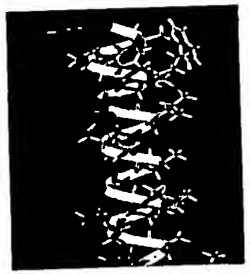


CRAMP (Mouse)



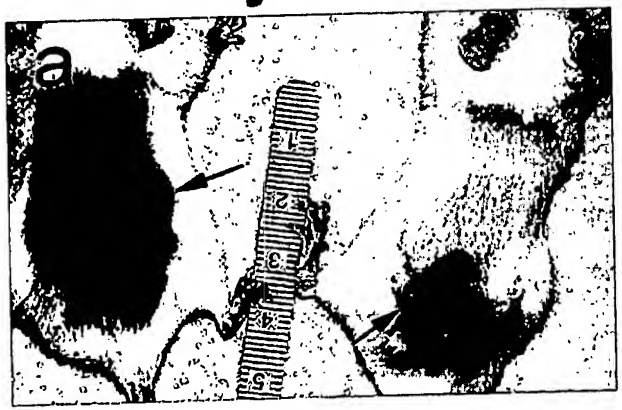


Similar Encoding Genes
Similar Alpha-Helical Structure
Similar Tissue Distribution
Similar Spectrum of Activity

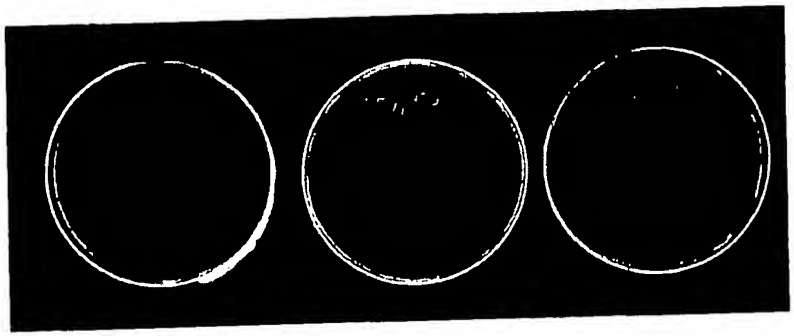


Cathelicidin deficient mice are more susceptible to Invasive Group A *Streptococcus*

+/+ day 7



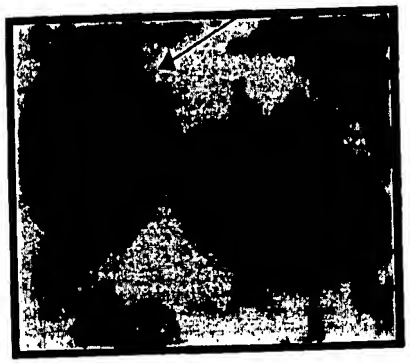
-/- day 7



Nature (2001) 414:6862

Activity against vaccinia supports role for Caths in immune defense against viral infection

No LL-37



LL-37 5 uM

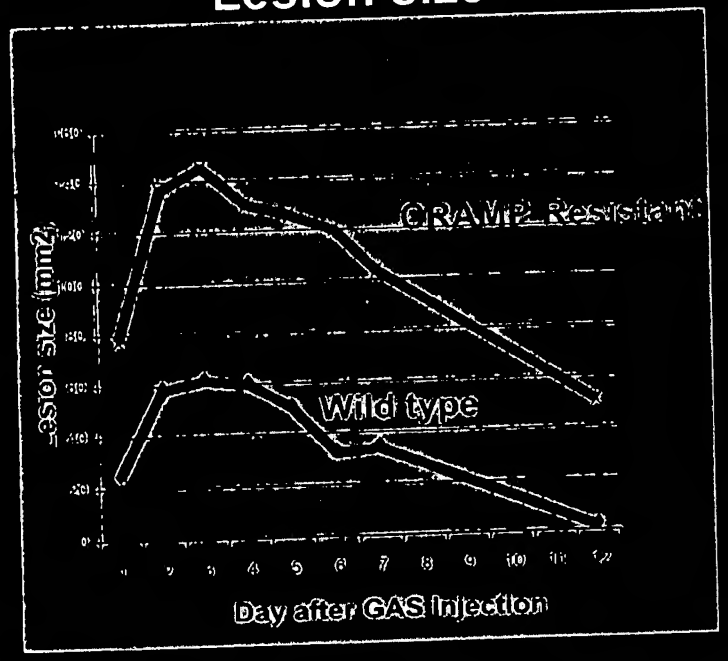
No Lesion 15/16 Control Mice

LL-37 (μM)	Altered virions/ virion number (%)	
0	1/23	(4%)
5	19/28	(67%)
25	27/30	(90%)

Vesicular Lesion 4/6 CRAMP --/--

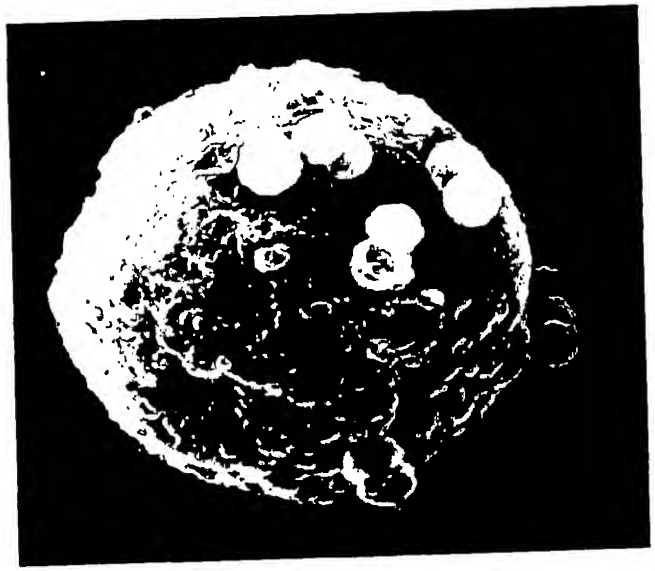
Bacteria (GAS) that are cathelicidin resistant are more pathogenic

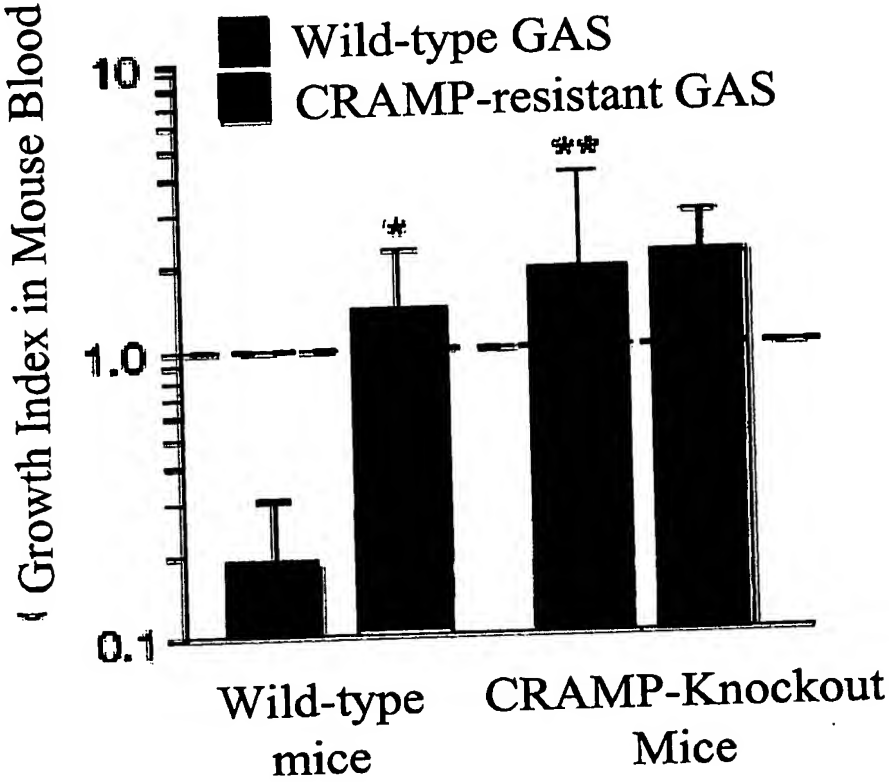
Lesion size



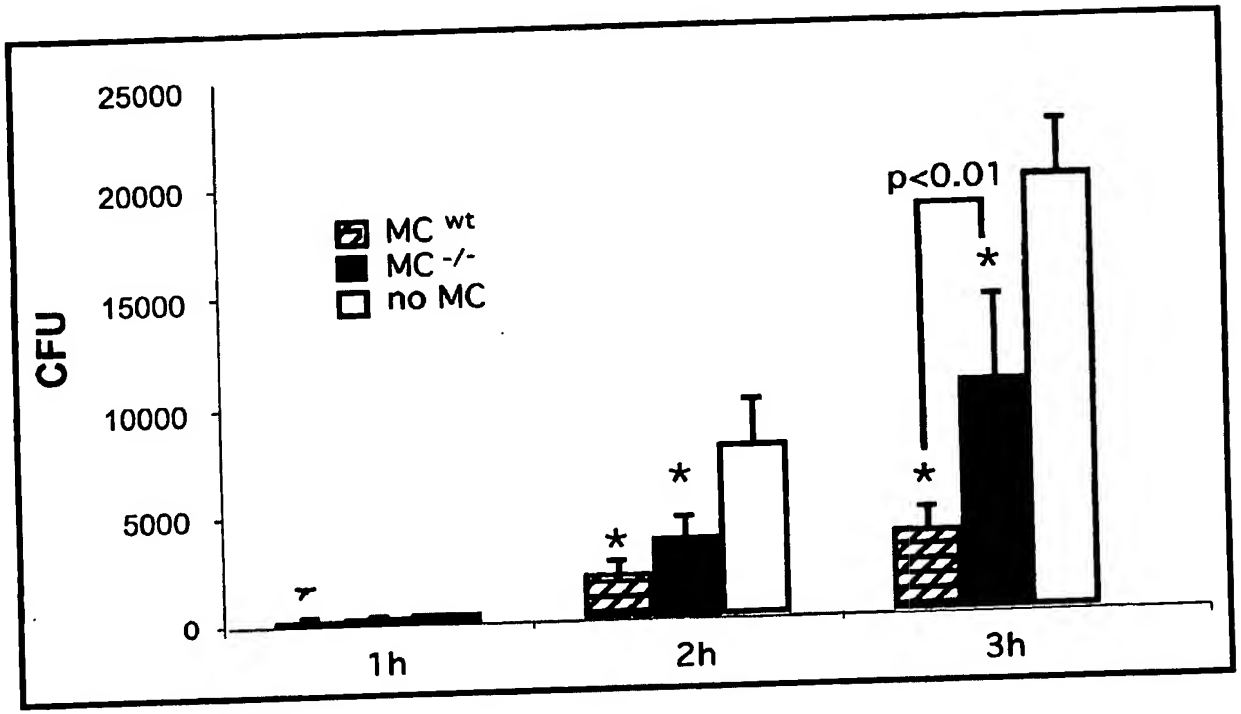
Nature, (2001) 414:454

Cramp Effect Evident in CELL-SPECIFIC assay
Blood-Killing Assay



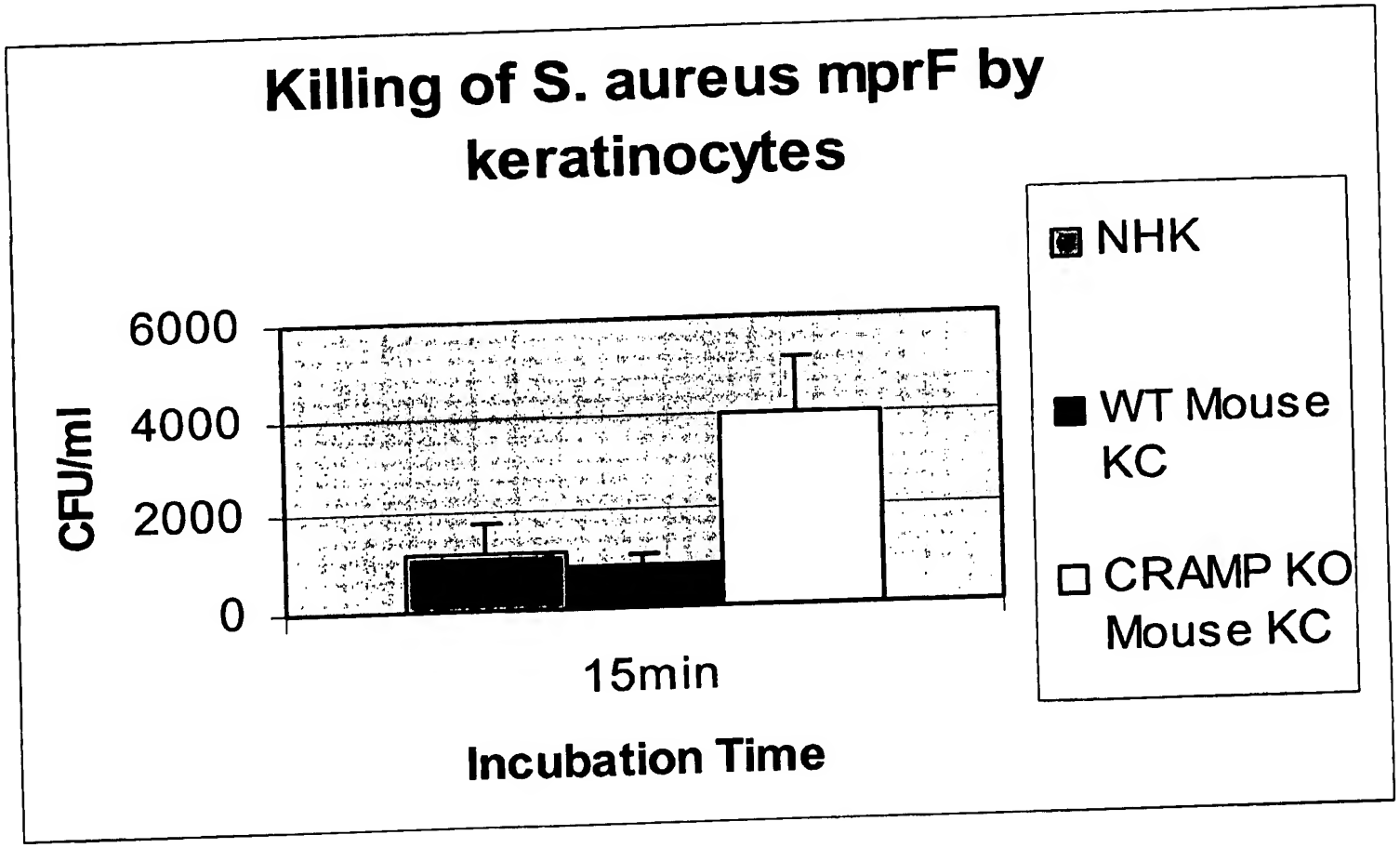


Cramp Effect Evident in CELL-SPECIFIC assay
Mast Cells



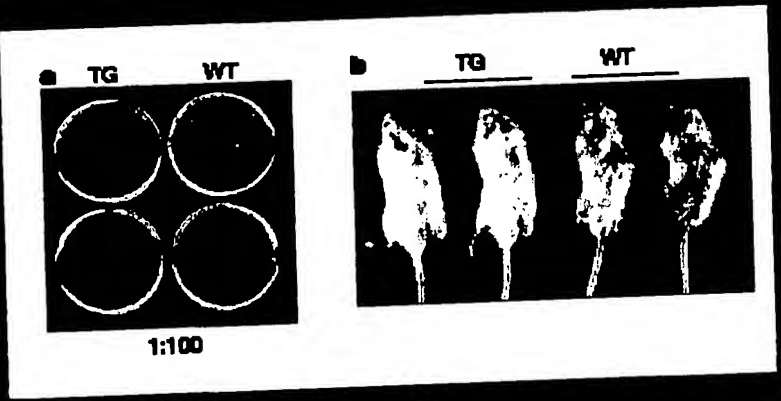
DiNardo et al. J Immunol (2003) 170:2274-78

Cathelicidin Effect Evident in CELL-SPECIFIC assay Keratinocytes



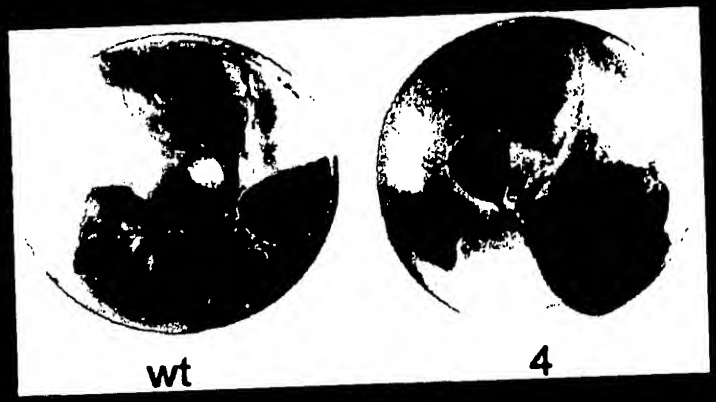
Immune protection by heterologous antimicrobial peptide over-expression.

Human antimicrobial peptide expressed in mice paneth cells confers resistance to *Salmonella typhimurium*



Nature, 2003 422 Apr 3

Amphibian antimicrobial peptide expressed in tabacum confers resistance to *Speudomonas syringae* or *P. nicotianae*



Biochem.J 2003, 370

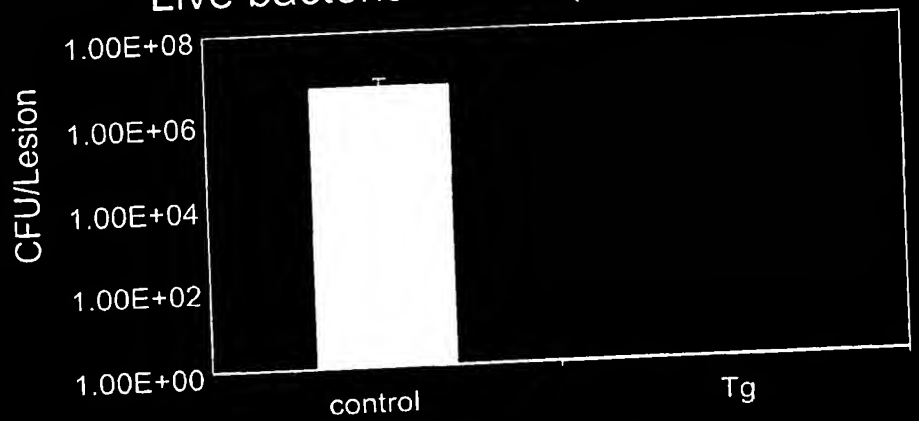
Immune protection by heterologous antimicrobial peptide over-expression.

PR-39 expression in skin

anti-PR-39 pre-immune

control Tg

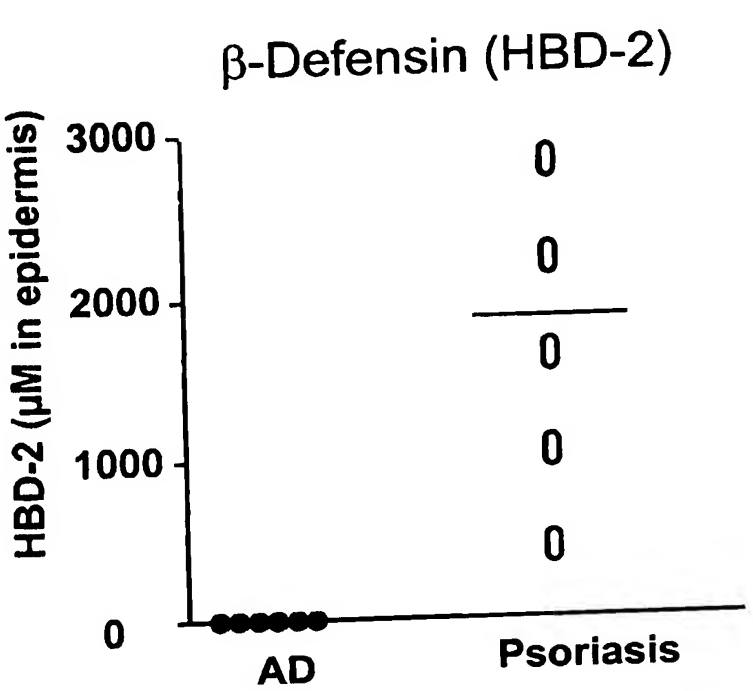
Live bacteria in skin (day6)

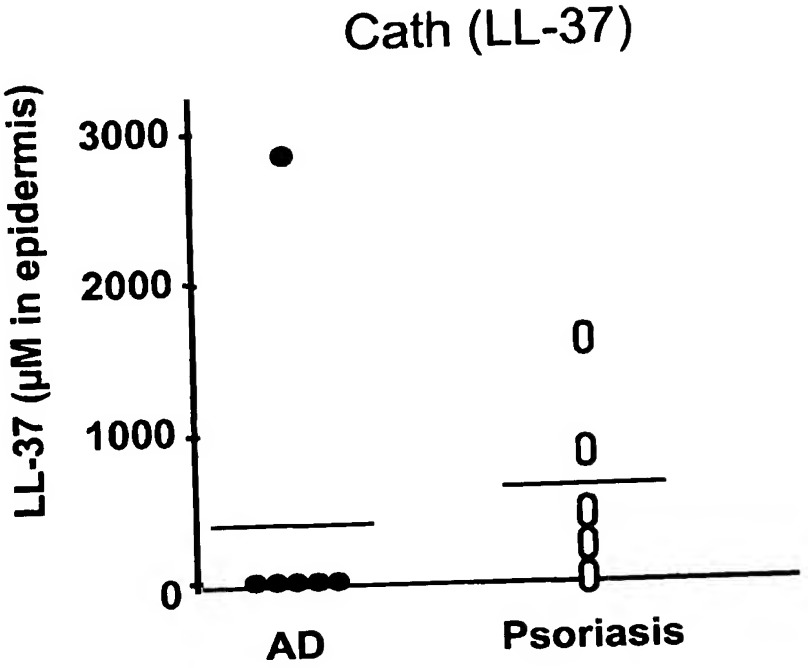


T. Ohtake

So why do the antimicrobial Peptides fail us?

Th2 cytokines in Atopic Dermatitis inhibit antimicrobial peptide induction





Ong et al. (2002) N Engl J Med 347:1151-60

Consequences of a lack of antimicrobial peptide expression?

Impetigo

Staph
Strep

Eczema Herpeticum

HSV, VZV

Eczema Vaccinatum

Cow pox

Recall
These peptides have diverse activity on the host.

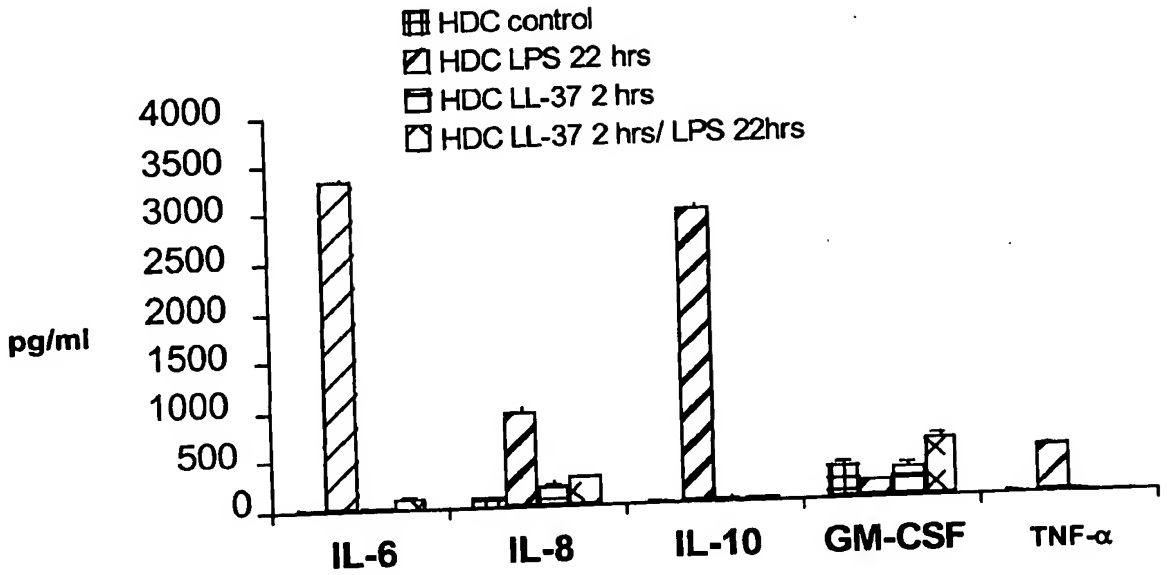
Caths

- Induce proteoglycans
  Gallo et al. (1994) PNAS
- Bind p47 Phox in NADPH oxidase
  Shi. et al. (1996) PNAS
- Bind FPRL1
  De Jang el al. (2000) J Exp Med.
- Inhibits proteasome
  Gao et al. (2000) J. Clin. Invest
- Angiogenic
  Li et al. (2000) Nature Medicine

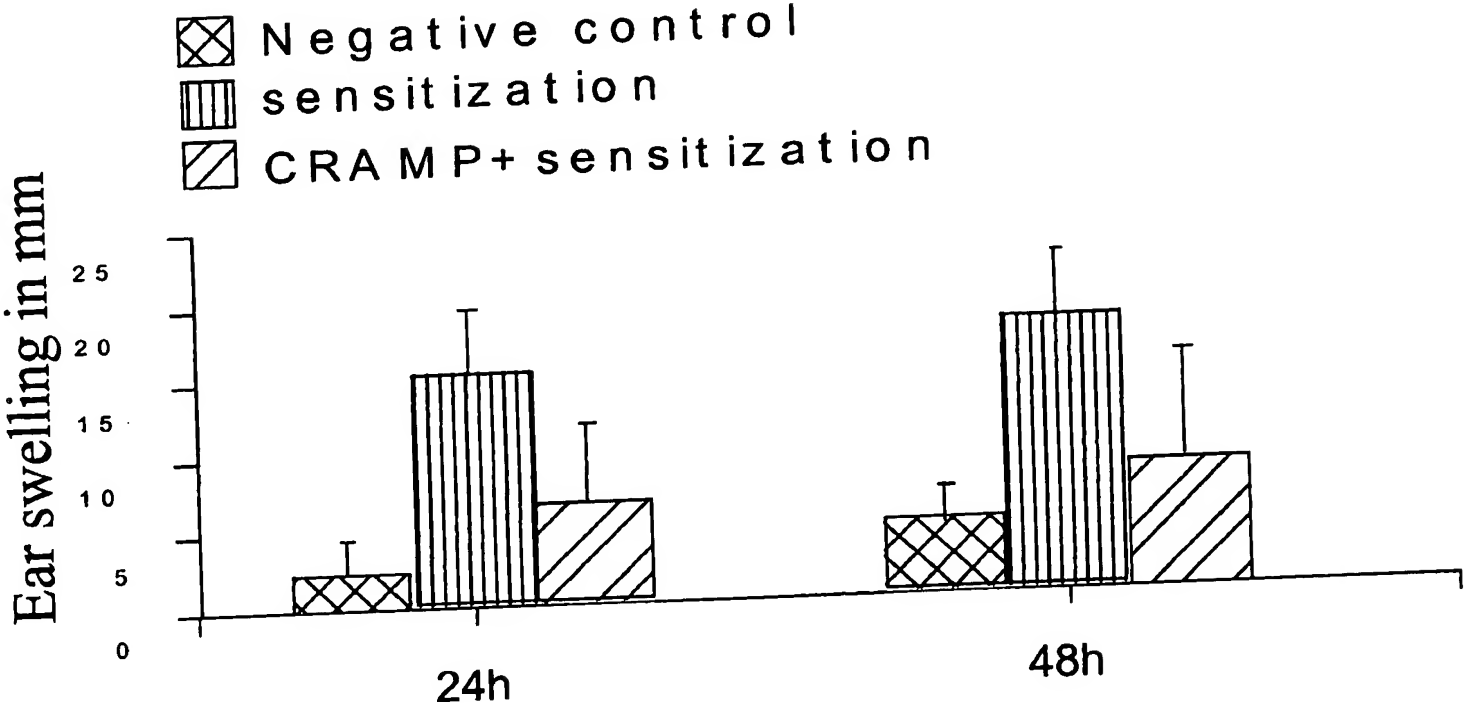
Defensins

- Binds CCR6
  Yang et al (1999) Science
- Activates TLR-4
  Biragyn et at. (2002) Science

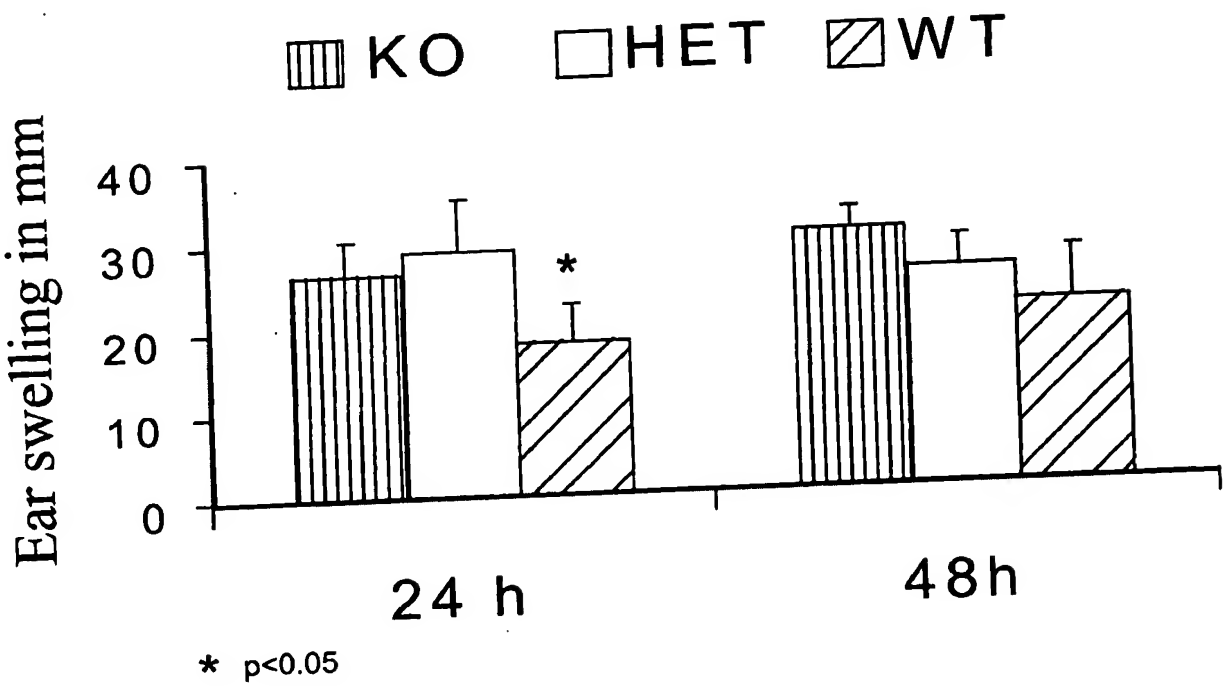
LL37 blocks LPS-induced chemokine release from Human Dendritic cells



CRAMP inhibits antigen presentation in vivo



CRAMP inhibits antigen presentation in vivo



Cathelicidins complex functions as a barrier in the skin

FUNCTIONS

Antimicrobial

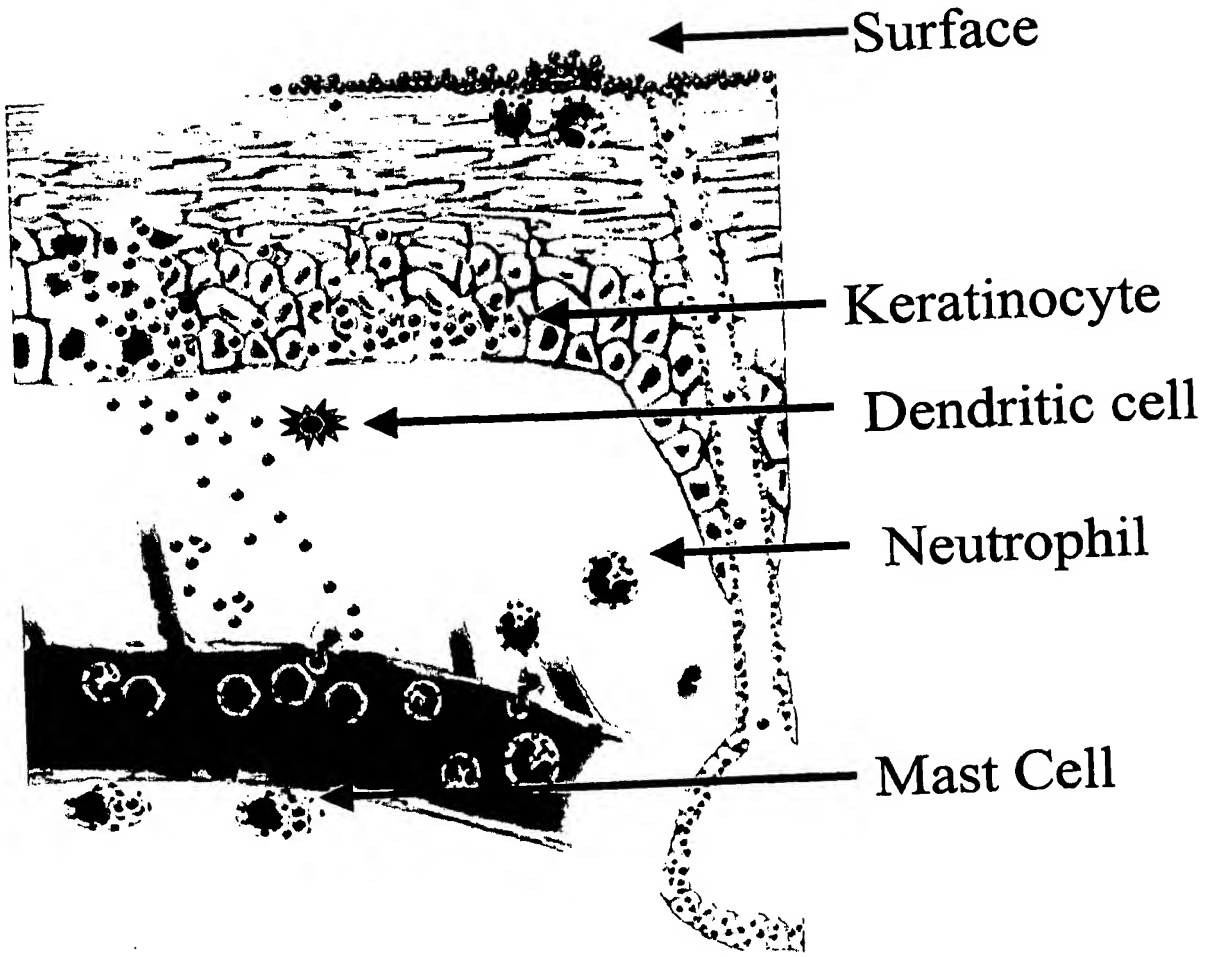
APC maturation

cytokine release

ECM synthesis

Chemotaxis

Angiogenic



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Donald Leung

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UCLA
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M. Murakami
Y. Kohgo

Boston, MA
Ken Huttner
Merton Bernfield

Inventor's Statement of Economic Interests in Potential Licensees

(Form TT-100, 1 July 1997, Modified by UCSD TechTIPS, 16 July 2002)

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TO BE COMPLETED BY ALO

Title of Invention: The Chip-Dasl Technology For Functional Genomics Studies

SD Case # : SD2003-244

Name of Inventor: Dr. Xiang-Dong Fu
Mail Code: MC: 0651
Department: Cellular Molecular Medicine
Campus: SD
Home Academic Unit: _____ (please complete)

Company Name: AVIVA Antibody Corporation
Address: 6160 Lusk Blvd., Suite C204
San Diego, CA 92121

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A. Are you a director, officer, trustee, board member, science advisor, or employee of, or do you hold any position of management in the company identified above?
____ No ____ Yes; if Yes, identify position: _____

B. Do you, or does your spouse or any dependent child:
1. Have, or expect to have, an investment or hold equity of $1,000 or more in the company identified above?
____ No ____ Yes; if Yes, $ _____ Value

2. Receive, have received, or expect to receive, income (including any payment, such as salary or consulting fee, or any loan or any gift) of $250 or more from the company identified above within the last or any 12 month period? (Do not include any salary paid by the University with funds provided by the company.)
____ No ____ Yes; if Yes, $ _____ Value

I have used all reasonable diligence in preparing this Statement and to the best of my knowledge it is true and complete.

Signature: _____ Date: _____

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International filing date: 20 October 2004 (20.10.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/512,953
Filing date: 21 October 2003 (21.10.2003)

Date of receipt at the International Bureau: 28 January 2005 (28.01.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

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